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(54) Title: ENZYMATICALLY ACTIVATED POLYMERIC DRUG CONJUGATES

(57) Abstract

The present invention relates to a polymeric drug conjugate with one or more biologically active agents conjugated via an enzymatically cleavable linker to either a regular repeating linear unit comprising a water soluble polymer segment and a multifunctional chemical moiety, or a branched polymer comprising two or more water soluble polymer segments each bound to a common multifunctional chemical moiety, as well as to methods of making such conjugates. The present invention is also directed to pharmaceutical compositions comprising such conjugates and to the use of such conjugates to treat pathological conditions.

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ENZYMATICALLY ACTIVATED POLYMERIC DRUG CONJUGATES

1. FIELD OF INVENTION

The present invention relates generally to polymeric drug conjugates composed of biologically active agents attached to regular repeating linear co-polymers or branched co-polymers by means of an enzymatically cleavable linker. In particular, the linker may contain one or more chemical bonds that are cleaved by enzymes, and, in some cases, may be further cleaved by changes in pH, ionic, or redox conditions. As such, the polymeric drug conjugates of the present invention can be specifically designed to provide for optimal enzymatic approach and cleavage of the linker by modifying the co-polymer. The invented polymeric drug conjugates may be used to modify drug solubility, drug bioavailability, drug residence time, drug absorption characteristics, and/or drug bioactivity.

2. BACKGROUND AND SUMMARY OF THE INVENTION

Novel formulation technologies have been developed to improve the delivery of many pharmaceutical agents, primarily to overcome issues of aqueous solubility, drug toxicity, bioavailability, and patient compliance (e.g., increase the time period between drug administrations). Drug delivery techniques can increase the therapeutic range of a compound by decreasing toxicity, thereby broadening the indications and clinical use of important drugs. In many cases, clinical indications of important pharmaceutical agents, particularly anti-cancer drugs, are often dose-limited because of systemic toxicity. For example, the toxicity of potentially important compounds can be significantly decreased by direct delivery of the active agent to specific tissues using implantable, bioresorbable polymers (Langer, 1990). However, implantable polymer technologies for drug delivery have inherent limitations, such as, for example, the fact that drug release is a function of hydrolytic degradation of the associated polymer or of simple diffusion of the drug from the polymer matrix, and therefore, the release characteristics are not a function of the disease being treated but are actually independent of the disease.

Likewise, much of the previously described and known formulation technologies have limitations which will preclude their use with many types of drugs or many disease indications, and in general are not site-specific delivery technologies. For example, a widely utilized

strategy for sustained delivery is to trap or encapsulate a drug into a lipid or polymer, limiting the availability of the agent to the biological system (Allen, *et al.*, 1992; Thierry, *et al.*, 1993; Tabata, 1993). In this case, the drug must either diffuse out of the capsule or polymer matrix, or the encapsulating agent must dissolve, disintegrate, or be absorbed before the drug can be released in a form which can be absorbed by the surrounding tissue. These techniques also rely on hydrolytic degradation for drug release. Polymer or lipid encapsulation systems do not have inherent properties which provide for site-specific (targeted) release of the encapsulated agent. In addition, polymer encapsulation systems are normally only suitable for water-soluble drugs, while liposomal formulations are restricted to those lipid soluble drugs which will partition in the liposomal bilayers without disruption of the bilayer integrity.

Macromolecules in the form of synthetic, natural, or semi-synthetic (chemically modified natural macromolecules) polymers have been utilized as carriers for a variety of pharmaceutical agents (*see* for example, Pachence and Kohn, 1998). Various chemical spacer groups have been previously used to covalently couple active agents to the polymer to create a conjugate capable of controlled or sustained release of a drug within the body. These spacer groups provide biodegradable bonds that permit controlled drug release. The size and nature of the spacer groups, and the charge and structure of the polymer are important characteristics to consider in the design of a drug with controlled or sustained release characteristics. As a result, the release of a drug often depends on hydrolytic cleavage of the bond between the polymer and active agent. However, while such a method does provide sustained release, it does not inherently target the active agent to a specific tissue.

Although polymeric drug conjugates can in general provide a method for controlled drug release or directed drug distribution in the body (and thereby improve the drug therapeutic index), the successful application of polymeric drug delivery systems depends to a great extent on: (1) the ability to reproducibly prepare well-defined polymer/drug conjugates; (2) providing an adequate payload (e.g., the ratio of drug molecular weight (MW) to polymer MW must be maximized); and (3) the choice of a linking group to attach the drug to the polymer. Particularly with natural and semi-synthetic polymers, covalent attachment of drugs to a polymer in general will lead to a random distribution along the polymer backbone. The spacing between each attached active agent is therefore also random, and in general not controllable.

It is well known that various proteolytic enzymes are produced in greater quantity by cells near or at the site of disease, or at the site of infection by microbes or host cells. For example, matrix metalloproteinases (MMPs) are a major family of enzymes which regulate extracellular matrix composition and modulate the interaction between cells and ECM (Massova, *et al.*, 1998). In addition to the normal role of MMPs in healing and metabolism, this enzyme family is also implicated in various pathological processes, including chronic inflammation, arthritis, and cancer. In particular, MMPs are active during tumor growth and have been found to be necessary for metastasis (Chambers and Matrisian, 1997).

Furthermore, numerous enzymes are produced by pathogens at the site of an infection, or by host cells (such as leukocytes) that are involved in combating infection. Thrombin-like alanine aminopeptidase and elastase-like enzymatic activity are known to be common in bacterial infections (Finlay and Cossart, 1997), and the amino acid cleavage sequences of such enzymes are well-documented.

Knowledge of enzyme production or up-regulation due to pathological conditions has been previously used as a strategy for drug delivery. For example, it has been shown that amino acid sequences known to be cleaved by specific enzymes present at the site of an infection can be coupled to an antibiotic, which can subsequently be incorporated into a polyvinyl alcohol hydrogel wound dressing (Suzuki, *et al.*, 1998). Antibiotics such as gentamicin can thus be selectively released into infected wound exudate. Likewise, enzymes which are produced by cancer cells, such as serine protease prostate-specific antigen, can be used to activate prodrugs (Denmeade, *et al.*, 1998).

More recently, a number of polymer/drug conjugates have been reported in the literature. Kopecek, *et al.* (U.S. Patents No. 5,037,883 and 5,258,453) describe the use of polymeric carriers attached to drugs with a linking chain that is cleaved by intracellular enzymes. This method, however, is limited by the necessity of the polymeric carrier-linking chain-drug conjugate to be taken into the cell before enzymatic cleavage can occur. Particularly, these patents describe a polymer with a targeting moiety wherein degradation of the drug-carrier linkage occurs via intracellular lysosomal hydrolysis. The technology of Kopecek further relies on chemically linking drugs to preformed polymers. This method limits the amount of drug bound to the polymer (typically less than 50% of the potential linking sites), and the resulting conjugate does not have a regularly repeating drug unit.

In a similar fashion, others have identified methods for releasing active agents coupled to polymers which rely on biodegradation of the bonds between the drug and the polymer. For example, Thorpe (U.S. Patent 5,474,765) describes a two component system consisting of a polyanionic polymer and a steroid linked via a hydrolyzing chemical bond. According to Thorpe, sulfated polyanionic polysaccharides (such as heparin) are used as the primary polymer constituent, and the use of synthetic organic sulfated polymers such as polystyrene sulfonate, sulfated polyvinyl alcohol, or polyethylene sulfonate is contemplated. The tissue-targeting component of the Thorpe invention is the endothelial cell-binding binding portion of heparin and similar polymers. According to Thorpe, the active agents are randomly conjugated to pre-formed polymers. Furthermore, these polymers are not inherently water soluble, nor are they taught to extend drug residence time. In addition, the biologically releasable bonds linking the active agent to the polymer in Thorpe, are generally hydrolyzable, and are not disease specific. Thorpe does not describe drug releasing conditions which would lead to tissue-localized high concentrations of the active agent.

Other groups have described alternating co-polymers of PEG that result in water-soluble polymers for drug delivery. For example, Zalipsky, *et al.* (U.S. Patents No. 5,219,564 and 5,455,027) describe a linear preformed polymer of PEG and the amino acid lysine to yield functional pendant groups (such as the terminal carboxyl group of lysine) at regular intervals. However, these methods do not describe methods of providing drug attachment along the polymer backbone at regular intervals. Likewise, the concept of enzymatically cleavable linking groups which would provide site-directed drug delivery is not disclosed.

Polymers, such as those described by Zalipsky, have been used by other authors to provide methods of drug release. For example, Huang, *et al.* (Bioconjugate Chem. 9:612-617, 1998) conjugates cysteine-containing peptides to a co-polymer of PEG-lysine (modified to provide regularly spaced thiol groups) using a disulfide linkage. Poiani, *et al.* (Bioconjugate Chem. 5:621-630, 1994; U.S Patents 5,372,807, 5,660,822, and 5,720,950) show the utilization of these same PEG-lysine with the anti-fibrotic compound cis-hydroxyproline. As with Zalipsky, neither Huang nor Poiani describe methods of providing drug attachment along the polymer at regular intervals, nor is the concept of metabolically cleavable linking groups described by these authors.

Other technologies provide methods for attaching polymers to active agents (particularly protein-based pharmaceutical compounds) as a means of creating a prodrug. For example, Greenwald, *et al.* (U.S. Patent No. 5,840,900) describe the covalent attachment of polyethylene glycol (PEG) to active agents to create prodrugs. Greenwald, however, describes a compound that relies on hydrolytic cleavage of large molecular weight PEG's (at least 20,000) to reconstitute the active agents. Small organic drugs would be inappropriate, as the ratio of PEG to drug would be too high. In addition, Greenwald does not consider the use of linking groups which are cleavable at the site of disease, and the resulting conjugate is not a regular polymer repeating structure.

A number of others (*e.g.* U.S. Patents No. 4,753,984, 5,474,765, 5,618,528, 5,738,864, 5,853,713) describe technologies which link active agents to pre-formed polymers, but are limited to a single class of active agents and do not describe methods for creating a regularly repeating polymer construct.

The present invention relates generally to polymeric drug conjugates composed of biologically active agents attached to regular repeating linear co-polymers or branched co-polymers by means of an enzymatically cleavable linker. More specifically, the present invention relates to a polymeric drug conjugate comprising one or more biologically active agents conjugated via an enzymatically cleavable linker to either a regular repeating linear unit comprising a water soluble polymer segment and a multifunctional chemical moiety, or a branched polymer comprising two or more water soluble polymer segments each bound to a common multifunctional chemical moiety. In a preferred embodiment, the one or more biologically active agents are conjugated via the linker to the multifunctional chemical moiety of the regular repeating linear unit. In another preferred embodiment, the one or more biologically active agents are conjugated via the linker to at least one of the two or more water soluble polymer segments.

In particular, the linker may contain one or more chemical bonds that may be cleaved by enzymes and, in some cases, additionally by changes in pH, ionic, or redox conditions, which are present in high concentration near, in, and /or on the surface of diseased tissues. More specifically, the linker may be further cleaved by hydrolysis, reduction reactions, oxidative reactions, pH shifts, photolysis, or combinations thereof. The linker may also be further cleaved by a non-specific enzyme reaction. In a preferred embodiment, the linker may

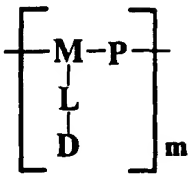
be cleaved by an intracellular or extracellular enzyme. Furthermore, in another preferred embodiment, the linker may be cleaved by a membrane-bound enzyme.

In another preferred embodiment, the linker is cleaved by an enzyme that is available at a target site, wherein the enzyme may also be up-regulated at the target site. Furthermore, the target site may be diseased tissue or biological fluid, wherein the diseased tissue may be present in skin, bone, cartilage, muscle, connective tissue, neural tissue, reproductive organs, endocrine tissue, lymphatic tissue, vasculature, or visceral organs and the biological fluid may be blood, pleural fluid, peritoneal fluid, joint fluid, pancreatic fluid, bile, or cerebral-spinal fluid.

In another preferred embodiment, the linker may be cleaved by any of an enzyme resulting from a microbial infection, a skin surface enzyme, or an enzyme secreted by a cell, by an enzyme secreted by a cancer cell, by an enzyme located on the surface of a cancer cell, by an secreted by a cell associated with a chronic inflammatory disease, by an enzyme secreted by a cell associated with rheumatoid arthritis, or by an enzyme secreted by a cell associated with osteoarthritis.

The conjugates of the present invention can be designed to provide for optimal enzymatic approach to and cleavage of the linker by modifying the water soluble polymer segments of the linear co-polymer and/or the multifunctional chemical moieties of the branched co-polymer. The polymeric drug conjugate of the present invention may be used to modify drug solubility, drug bioavailability, drug residence time, drug absorption characteristics, and/or drug bioactivity. General structures of the polymeric drug conjugates are shown below.

General Polymeric Drug Conjugate Formulas



poly[D-L-M-P]

Regular repeating linear polymeric drug conjugate

Branched polymeri drug conjugate

The copolymer backbones of the conjugate of the present invention are composed of water soluble polymer segments attached to multifunctional chemical moieties to form either regular linear repeating or branched scaffolds. These scaffolds are designed to provide a series of evenly spaced chemical functionalities for the attachment of biologically active moieties through an enzymatically cleavable linking group. The composition of the co-polymer can be modified to allow for optimal enzymatic approach to the enzymatically cleavable linkers by the modification of the size or chemical environment of the individual polymer segments and/or the multifunctional chemical moieties.

General Construct Formula **poly[D-L-M-P]**

The polymer construct **poly[D-L-M-P]** consists of a multifunctional chemical moiety, **M**, that is used to join water soluble polymer segments, **P**, to form a regular repeating linear co-polymer backbone and additionally to provide the chemical substituents for the attachment of a biologically active agent, **D**, via an enzymatically cleavable linker, **L**. The number of **M-P** repeats of the regular repeating linear polymer is designated by **m** wherein **m** is preferably an integer that is greater than or equal to 2, more preferably an integer from about 2 to about 25.

The water soluble polymeric drug conjugate can be designed to increase the water solubility of **D** and be formulated to be administered through injection, oral, topical, inhalation delivery, subcutaneous deposition (implant), deposition using minimally invasive surgical pr cedures such as laproscopy, or other previously described physical delivery methods.

The pharmaceutical agent released from the co-polymer/linking agent conjugate by enzymatic activity provides reconstituted pharmaceutical activity in a high local tissue concentration. In general, the metabolically sensitive linking group, **L**, is designed to be cleaved by enzymes that are present at high concentration (higher than non-pathological levels) at or near the targeted disease site.

General Construct Formula $Q(-P-L-D)_k$

The branched polymeric drug conjugate of the formula $Q(-P-L-D)_k$ consists of a common multifunctional chemical moiety, **Q**, that is used to attach **k** number of water soluble polymer segments, **P**, attached to a biologically active agent, **D**, via an enzymatically cleavable linker, **L**. The water soluble polymeric drug conjugate can be designed to increase the water solubility of **D** and can be formulated to be administered through injection, oral, topical, inhalation delivery, subcutaneous deposition, deposition using minimally invasive surgical procedures (*e.g.* laproscopy), or other previously described physical delivery methods.

The pharmaceutical agent released from the polymeric conjugate by enzymatic activity provides reconstituted pharmaceutical activity in a high local tissue concentration. In general, the metabolically sensitive linking group, **L**, is designed to be cleaved by enzymes that are present at high concentration (higher than non-pathological levels) at or near the site of disease.

The present invention also includes pharmaceutical compositions comprising the novel conjugate and a physiologically acceptable carrier, wherein the pharmaceutical composition is preferably suitable for injection, or oral, topical, inhalation, or implantation methods of administration.

The present invention also includes methods of alleviating a pathological condition comprising administering an effective amount of novel conjugates of the present invention, wherein the pathological condition may include neoplastic diseases, chronic inflammatory diseases acute inflammatory diseases, cardiac diseases, renal diseases, liver diseases, lung diseases, neurological diseases, musculoskeletal diseases, and immunological disorders. Furthermore, the method may include regulating cardiac function, renal function, liver

function, lung function, or neurological function; modulating immunological function; modulating hormonal function; treating microbial infections; or regulating scar tissue.

The present invention provides novel compositions for creating tissue-targeting formulations consisting of pharmaceutical agents conjugated to water soluble polymers. The present invention provides a method of targeting drug release by using chemical linking groups between the polymer and the pharmaceutical agent that can be specifically cleaved at the site of disease. In addition, the pharmaceutical agents according to the present invention are spaced along the water soluble polymer backbone at regular intervals, wherein the interval between each active agent is controlled by synthetic methods.

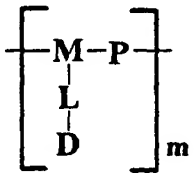
As a result, a unique aspect of the conjugate of the present invention is a construct which consists of a drug and linker which repeats on a water soluble polymer backbone. The spacing between attachment of the drug/linker group complex can be controlled by the synthetic methods presented herein. One preferred method of forming the conjugate of the present invention, is that the drug, linker, and monomer be conjugated first, and then the resulting product is coupled to a water soluble polymer which then forms the polymer conjugate. This provides for a high degree of drug-linker substitution on the polymer construct (typically greater than 90%), providing a regular repeating unit of the drug/linker along the polymer backbone.

The present invention also requires that the linking group be cleavable by enzymatic activity, such as by enzymes which may be present in high concentrations near, in and/or on the surface of diseased tissue. This unique aspect provides a mechanism for obtaining target site-directed drug delivery.

3. DETAILED DESCRIPTION OF THE INVENTION

This invention describes polymeric drug conjugates formed by covalently attaching a biologically active agent to a co-polymeric backbone via an enzymatically cleavable linker. The conjugates **poly[D-L-M-P]** and **Q(-P-L-D)$_k$** are administered to a patient, wherein the biologically active agent is released from the polymer backbone by a physiological process, and the agent's activity is reconstituted. The linker consists of chemical chains with one or more bonds that are susceptible to physiological cleavage, preferably enzymatic cleavage. The structures of the invented constructs are shown in General Polymeric Drug Conjugate Formulas.

General Polymeric Drug Conjugate Formulas



poly[D-L-M-P]

Regular repeating linear polymeric drug conjugate

Branched polymeric drug conjugate

General Construct Formula **poly[D-L-M-P]**

The invented polymer construct of formula **poly[D-L-M-P]** consists of a multifunctional chemical moiety , **M**, that is composed of organic compounds, amino acids, or a combination of both which contains chemical functionalities that can be used to form covalent bonds with the polymer segments, **P**, and the linking group, **L**; **L** is a "linker" that can consist of, either independently or in combination, amino acids, sugars, nucleic acids, or other organic compounds which possess one or more chemical bonds that are enzymatically cleavable; **D** is a biologically active agent which possesses a chemical substituent that can form a covalent bond to the linking group, **L**; **P** is a water soluble polymer or copolymer that contains at least two functionalities that can form covalent chemical bonds to substituents **n**

the monomer, **M**; and **m** is the number of polymer repeats, typically ranging from 5 to 12 but can extend over the range of 2-25.

In a preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, the multifunctional chemical moiety comprises N-(2-hydroxyacetyl)serine, the linker comprises (H-Leu-Gly-Pro-Ala-NH-CH_2-CH_2-NH_2), and the biologically active agent comprises doxorubicin-14-O-hemiglutarate.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 1,000, the multifunctional chemical moiety comprises tris(2-aminoethyl)amine, the linker comprises (H-Val-Pro-Arg-OH), and the biologically active agent comprises N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic (1→6)-disulfide.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, the multifunctional chemical moiety comprises N_α-(-*p*-aminophenylacetyl)-*p*-aminophenylalanyl hydrazide, the linker comprises (OCH-CO-Gly-Pro-Leu-Gly-Pro-OH), and the biologically active agent comprises a pharmaceutical analog of Leu-Gly-α-5-fluorouracil.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 1,000, the multifunctional chemical moiety comprises 3,5-dihydroxyphenylacetic acid, the linker comprises (H-Cys(S-CH_2-CH_2-CO-NH-CH_2-CH_2-NH_2)-Glu-Glu-Glu-OH), and the biologically active substance comprises a pharmaceutical analog of Leu-Gly-α-5-fluorouracil.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, the multifunctional chemical moiety comprises lysine, the linker comprises (H-Gly-Pro-Tyr-Ala-Tyr-Trp-Lys-NH_2), and the biologically active agent comprises methotrexate.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, the multifunctional chemical moiety comprises lysine, the linker comprises (H-Gly-Pro-Lys-Pro-Val-Gly-Nva-Trp-Lys-OH), and the biologically active agent comprises methotrexate.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, the multifunctional chemical

moiety comprises lysine, the linker comprises (H-Gly-Pro-Leu-Gly-Pro-Lys-NH_2), and the biologically active agent comprises methotrexate.

General Construct Formula $Q(-P-L-D)_k$

The invented polymer construct of formula $Q(-P-L-D)_k$ consists of a common multifunctional chemical moiety, **Q**, that is used to attach **k** number of water soluble polymer segments, **P**, and a n enzymatically cleavable linker, **L**, that connects **P** to **D**, a biologically active agen, wherein **k** is preferably an integer from 1 to about 100, more preferably an integer from 2 to about 100.

The water soluble polymer conjugate prodrug system can be designed to increase the water solubility of **D** and can be formulated to be administered through injection, oral, topical, inhalation delivery, subcutaneous deposition, deposition using minimally invasive surgical procedures such as laproscopy, or other previously described physical delivery methods.

Another aspect of the invention is that the polymer construct $Q(-P-L-D)_k$ allows for multiple equally spaced drug-linker substituents on each common multifunctional chemical moiety, **Q**. The structure, chemical composition, or size of the polymer segment, **P**, can be easily changed to allow for a facile approach of the enzyme to the enzymatically cleavable linker, **L**, and optimize the biological utility of the product construct.

The pharmaceutical agent released from the polymer/linking agent conjugate by metabolic activity would provide reconstituted pharmaceutical activity in a high tissue local concentration.

In a preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 4,000, the common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, the linker comprises (H-Gly-Pro-Leu-Gly-Pro-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active agent comprises methotrexate, and said integer is 4.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 4,000, the common multifunctional chemical moiety comprises an 8 arm dendrimer, the linker comprises (H-Gly-Pro-Leu-Gly-Pro-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active agent comprises methotrexate, and the integer is 8.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, the common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, the linker comprises (H-Glu-Glu-Glu-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active agent comprises methotrexate, and the integer is 4.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, the common multifunctional chemical moiety comprises an 8 arm dendrimer, the linker comprises (H-Glu-Glu-Glu-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active agent comprises methotrexate, and the integer is 8.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, the common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, the linker comprises (H-Gly-Pro-Lys-Pro-Val-Gly-Nva-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active substance comprises methotrexate, and the integer is 4.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, the common multifunctional chemical moiety comprises an 8 arm dendrimer, the linker comprises (H-Gly-Pro-Lys-Pro-Val-Gly-Nva-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active agent comprises methotrexate, and the integer is 8.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, the common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, the linker comprises (H-Gly-Pro-Tyr-Ala-Tyr-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active agent comprises methotrexate, and the integer is 4.

In another preferred embodiment, the water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, the common multifunctional chemical moiety comprises 8 arm dendrimer, the linker comprises (H-Gly-Pro-Tyr-Ala-Tyr-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), the biologically active agent comprises methotrexate, and the integer is 8.

Assembly of the Polymeric Drug Conjugates

Polymeric drug conjugate poly[D-L-M-P] of this invention can be assembled using four separate units; the multifunctional chemical moiety, **M**, an enzymatically cleavable linker, **L**, a biologically active agent, **D**, and a water soluble polymer segment, **P**. These individual units are initially substituted with one or more reactive functional groups that are used to form stable chemical bonds with the other units of the construct.

According to the present invention, 1 to 100% of the multifunctional chemical moieties, **M**, incorporated into the copolymer backbone may be substituted with the biologically active agent-linker conjugate, **D-L**. Furthermore, the chemical linkage connecting the water soluble polymer segment, **P**, to the multifunctional chemical moiety, **M**, may include a carbamate, thiocarbamate, ether, urea, thiourea, carbonate, thiocarbonate, or ester function

Construct $Q(\text{-}P\text{-}L\text{-}D)_k$ of this invention can also be assembled using four units; the common multifunctional chemical moiety, **Q**, an enzymatically cleavable linker, **L**, a biologically active agent, **D**, and a water soluble polymer segment , **P**. These individual units are initially substituted with one or more reactive functional groups that are used to form stable bonds with the other units of the construct.

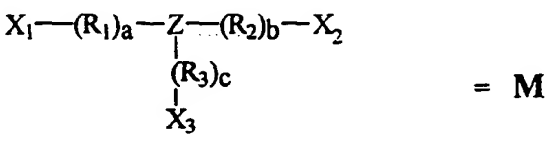
According to the present invention, 1 to 100% of the water soluble polymer segments, **P**, incorporated onto the common multifunctional chemical moiety, **Q**, may be substituted with the biologically active agent-linker conjugate, **D-L**. Furthermore, the chemical linkage connecting the water soluble polymer segment, **P**, to the common multifunctional chemical moiety, **Q**, or the linker, **L**, may include a carbamate, thiocarbamate, ether, urea, thiourea, carbonate, thiocarbonate, or ester function.

Description of the Units of the Polymeric Drug Conjugates

The multifunctional chemical moiety, **M**

The multifunctional chemical moiety, **M**, is designed to covalently join the water soluble polymer segments, **P**, and also bind the linker, **L**. It is designed and synthesized to provide the structure and chemical functionalities illustrated in General Formula 1.

General F rmula 1



where X_1, X_2, and X_3 are chemical substituents that can be used to form covalent bond with the polymer segments, **P**, or the enzymatically cleavable linker, **L**. X_1, X_2, and X_3 can independently be or be derived from, but are not restricted to, hydroxyl, amino, thiol, alkyl or aryl disulfide, isothiocyanate, thiocarbonylimidazole, thiocarbonylchloride, aldehyde, ketone, carboxylic acid, carboxylic acid ester, sulfonic acid, sulfonic acid ester, sulfonyl chloride, phosphoric acid, alkyl or aryl succinimidyl carbonate, alkyl or aryl chlorocarbonate, alkyl or aryl succinimidylthiocarbonate, alkyl or aryl chlorothiocarbonate, halide, or thioester functions (possibly substituted with appropriate protecting groups that can be removed before further chemical reaction). R_1, R_2, or R_3 act as spacers that initially separate the reactive functional groups to provide an optimal chemical and steric environment for the assembly of the final polymeric drug conjugate, and ultimately separate the polymer segments, **P**, and linker , **L**, to allow for optimal biological activity of the invented construct., R_1, R_2, or R_3 can independently consist of, but are not restricted to, saturated and unsaturated, straight and branched alkyl, aryl, alkylaryl, heteroalkyl, heteroaryl, or heteroalkyaryl chains which may contain up to 20 carbon atoms. a = 0-2, b = 0-2, c = 0-2; and Z = C, CH,N, P, PO, aryl, or heteroaryl.

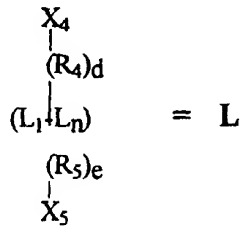
In a preferred embodiment, the multifunctional chemical moiety is derived from N-(2-hydroxyacetyl)serine, lysine, tris(2-aminoethyl)amine, N-(*p* –nitrophenylacetyl)-*p*-nitrophenylalanine acid hydrazide, 3,5-dihydroxyphenylacetic acid, 3,5-diaminobenzoic acid, or 6-amino-4-(2-aminoethyl)hexanoic acid.

The Linker, L

The enzymatically cleavable linker **L**, is illustrated in General Formula 2, where X_4 and X_5 are chemical substituents that can be used to form covalent bonds with the multifunctional chemical moiety, **M**, and the biologically active agent, **D**. X_4 and X_5 can independently be or be derived from, but are not restricted to, hydroxyl, amino, thiol, alkyl or aryl disulfide, isothiocyanate, thiocarbonylimidazole, thiocarbonylchloride, aldehyde, ketone, carboxylic acid, carboxylic acid ester, sulfonic acid, sulfonic acid ester, sulfonyl chloride, phosphoric acid, alkyl or aryl succinimidyl carbonate, alkyl or aryl chlorocarbonate, alkyl or aryl

succinimidylthiocarbonate, alkyl or aryl chlorothiocarbonate, halide, or thioester functions (possibly substituted with appropriate protecting groups that can be removed before further chemical reaction).

General Formula 2



R_4 or R_5 act as spacers that initially separate the reactive functional groups to provide an optimal chemical and steric environment for the assembly of the final polymeric drug conjugate, and ultimately separate the linker, **L**, from the biologically active agent, **D**, and the multifunctional chemical moiety, **M**, to allow for optimal biological activity of the invented construct. R_4, or R_4 can independently consist of, but are not restricted to, saturated and unsaturated, straight and branched alkyl, aryl, alkylaryl, heteroalkyl, heteroaryl, or heteroalkyaryl chains which may contain up to 20 carbon atoms. d = 0-2, e = 0-2; and $(L_1\text{-}L_n)$ is a chain consisting of, either independently or in combination, amino acids, sugars, nucleic acids, or other organic compounds which possess a single or multiple enzymatically cleavable bonds.

In a preferred embodiment, the linker may include an amino acid, a sugar, a nucleic acid, or other organic compounds, or combinations thereof. In another preferred embodiment, the linker may include a peptide sequence.

In a preferred embodiment, the peptide sequence may be cleaved by a serine protease such as thrombin, chymotrypsin, trypsin, elastase, kallikrein, or substilisin. Furthermore, the thrombin-cleavable peptide sequence may include -Gly-Arg-Gly-Asp-, -Gly-Gly-Arg-, -Gly-Arg-Gly-Asp-Asn-Pro-, -Gly-Arg-Gly-Asp-Ser-, -Gly-Arg-Gly-Asp-Ser-Pro-Lys-, -Gly-Pro-Arg-, -Val-Pro-Arg-, or -Phe-Val-Arg-. The elastase-cleavable peptide sequence may include -Ala-Ala-Ala-, -Ala-Ala-Pro-Val-, -Ala-Ala-Pro-Leu-, -Ala-Ala-Pro-Phe-, -Ala-Ala-Pro-Ala-, or -Ala-Tyr-Leu-Val-.

In another preferred embodiment, the linker may include a peptide sequence which

may be cleaved by a cysteine proteinase such as papain, actinidin, bromelain, lysosomal cathepsins, cytosolic calpain, and parasitic protease. Furthermore, the parasitic protease may be derived from *Trypanosoma* or *Schistosoma*.

In another preferred embodiment, the linker may include a eptide sequence which may be cleaved by an aspartic proteinase such as pepsin, chymosin, lysosomal cathepsins D, a processing enzyme, a fungal protease, and a viral proteinase. Furthermore, the processing enzyme may include renin, the fungal protease may include penicillopepsin, rhizopuspepsin, or endothiapepsin, and the viral protease may include the protease from the AIDS virus.

In another preferred embodiment, the linker may include a peptide sequence that can be cleaved by a matrix metalloproteinase such as collagenase, stromelysin, and gelatinase. Furthermore, the matrix metalloproteinase may include -Gly-Pro-Y-Gly-Pro-Z-, -Gly-Pro-Leu-Gly-Pro-Z-, -Gly-Pro-Ile-Gly-Pro-Z-, or -Ala-Pro-Gly-Leu-Z-, where Y and Z are amino acids. Preferably, the matrix metalloproteinase includes -Leu-Gly-, or Ile-Gly. Furthermore, the collagenase-cleavable peptide sequence may include -Pro-Leu-Gly-Pro-D-Arg-Z-, -Pro-Leu-Gly-Leu-Leu-Gly-Z-, -Pro-Gln-Gly-Ile-Ala-Gly-Trp-, -Pro-Leu-Gly-Cys(Me)-His-, -Pro-Leu-Gly-Leu-Trp-Ala-, -Pro-Leu-Ala-Leu-Trp-Ala-Arg-, or -Pro-Leu-Ala-Tyr-Trp-Ala-Arg-, where Z is an amino acid; the stromelysin-cleavable peptide sequence may include -Pro-Tyr-Ala-Tyr-Trp-Met-Arg-; and the gelatinase-cleavable peptide sequence may include -Pro-Leu-Gly-Met-Trp-Ser-Arg-.

In another preferred embodiment, the linker may include a peptide sequence that can be cleaved by an angiotensin converting enzyme, wherein the angiotensin converting enzyme may include -Asp-Lys-Pro-, -Gly-Asp-Lys-Pro-, or -Gly-Ser-Asp-Lys-Pro-.

In another preferred embodiment, the linker may include a peptide sequence that can be cleaved by a prostate specific antigen or a prostate specific membrane antigen, wherein the linker may include $-(Glu)_n-$, where n is an integer from 1 to 10.

The biologically active agent, **D**

The biologically active agent, **D**, consists of any biologically useful agent, analog, or metabolite, or a mixture of several systems, which possess (or can be modified to possess) at least one chemical functionality (for example, including, but not limited to, hydroxyl, amino, thiol, alkyl or aryl disulfide, isothiocyanate, thiocarbonylimidazole, thiocarbonylchloride,

aldehyde, ketone, carboxylic acid, carboxylic acid ester, sulfonic acid, sulfonic acid ester, sulfonyl chloride, phosphoric acid; alkyl, aryl succinimidyl carbonate, alkyl or aryl chlorocarbonate, alkyl or aryl succinimidylthiocarbonate, alkyl or aryl chlorothiocarbonate, halide, or thioester) for covalent attachment to the linker, L.

Biologically active agents that may be delivered by the system of the present invention include, but are not restricted to, analgesics, anesthetics, antifungals, antibiotics, antiinflammatories, anthelmintics, antidotes, antiemetics, antihistamines, antihypertensives, antimalarials, antimicrobials, antipsychotics, antipyretics, antiseptics, antiarthritics, antituberculotics, antitussives, antivirals, cardioactive drugs, cathartics, chemotherapeutic agents, corticoids (steroids), antidepressants, depressants, diagnostic aids, diuretics, enzymes, expectorants, hormones, hypnotics, minerals, nutritional supplements, parasympathomimetics, potassium supplements, radiation sensitizers, sedatives, sulfonamides, stimulants, sympathomimetics, tranquilizers, urinary antiinfectives, vasoconstrictors, vasodilators, vitamins, xanthine derivatives, and the like. The biologically active agents could be small organic molecules, naturally isolated entities or their analogs, or peptide based drugs. The biologically active agents may also comprises DNA, RNA, a DNA fragment, an RNA fragment, or a plasmid.

In a preferred embodiment, the chemotherapeutic agent comprises a nitrogen mustard, an ethylenimine, a methylmelamine, a nitrosourea, an alkyl sulfonate, a triazene, a folic acid analog, a pyrimidine analog, a purine analog, a vinca alkaloid, an epipodophyllotoxin, an antibiotic, an enzyme, a biological response modifier, a platinum complex, a methylhydrazine derivative, an adrenocorticol suppressant, a somatostatin, a somatostatin analog, a hormone, a hormone antagonist, or combinations thereof. Preferrably, the chemotherapeutic agent comprises methotrexate, taxol, aminopterin, doxorubicin, bleomycin, camptothecin, etoposide, estramustine, prednimustine, melphalan, hydroxyurea, or 5-fluorouracil.

In another preferred embodiment, the peptide based pharmaceutical agent comprises a cytokine, a growth factor, a cell receptor antagonist, or a cell receptor agonist.

In another preferred embodiment, the biologically active agent comprises an eptifibatide and other platelet binding proteins, a granulocyte colony stimulating factor, a human growth factor, a vascular endothelial growth factor, a bone morphogenic protein, an interferon, or an interleukin.

The water soluble polymer segment, **P**

The water soluble polymer segment, **P**, consists of is a relatively short, water soluble polymeric system (for example, average M.W. 400-25,000) which contains at least two chemical functionalities (for example, including but not limited to, hydroxyl, amino, thiol, alkyl or aryl disulfide, isothiocyanate, thiocarbonylimidazole, thiocarbonylchloride, aldehyde, ketone, carboxylic acid, carboxylic acid ester, sulfonic acid, sulfonic acid ester, sulfonyl chloride, phosphoric acid, alkyl or aryl succinimidyl carbonate, alkyl or aryl chlorocarbonate, alkyl or aryl succinimidylthiocarbonate, alkyl or aryl chlorothiocarbonate, halide, or thioester) that can be used for covalent attachment to the multifunctional chemical moiety, **M**. **P** can be, but is not restricted to, poly(ethylene glycol), poly(vinyl alcohol), poly(2-hydroxyethyl methacrylate), poly(acrylic acid), poly(methacrylic acid), poly(maleic acid), poly(lysine), and the like or a copolymer consisting of a mixture of these or other polymeric entities possibly substituted with organic functional groups.

The common multifunctional chemical moiety, **Q**

The common multifunctional chemical moiety, **Q**, is designed to couple to **k** number of short water soluble polymer segments, **P**. It is designed and synthesized to provide the structure and chemical functionalities illustrated in General Formula 3.

General F rmula 3

$$J(-X_6)_k$$

where X_6 is a chemical substituent that can be used to form covalent bonds with the polymer segments, **P**. X_6 can independently be or be derived from, but are not restricted to, hydroxyl,

amino, thiol, alkyl or aryl disulfide, isothiocyanate, thiocarbonylimidazole, thiocarbonylchloride, aldehyde, ketone, carboxylic acid, carboxylic acid ester, sulfonic acid, sulfonic acid ester, sulfonyl chloride, phosphoric acid, alkyl or aryl succinimidyl carbonate, alkyl or aryl chlorocarbonate, alkyl or aryl succinimidylthiocarbonate, alkyl or aryl chlorothiocarbonate, halide, or thioester functions (possibly substituted with appropriate protecting groups that can be removed before further chemical reaction). **J** acts as a spacer that initially separate the reactive functional groups to provide an optimal chemical and steric environment for the assembly of the final polymeric drug conjugate, and ultimately separate the polymer segments, **P**, to allow for optimal biological activity of the invented construct., **J** can independently consist of, but is not restricted to, saturated and unsaturated, straight and branched alkyl, aryl, alkylaryl, heteroalkyl, heteroaryl, or heteroalkyaryl chains which may contain up to 100 carbon atoms, preferably 20.

In a preferred embodiment, the common multifunctional chemical moiety includes pentaerythritol, dendrimers, or branched lysine trees.

Polymeric Drug Conjugate Assembly Pathways

There are several synthetic pathways that can be used to assemble the invented polymeric drug conjugates described herein.

Construct, **poly[D-L-M-P]**

Assembly Method I

The method of assembly, illustrated in Reaction Scheme 1, requires an initial covalent coupling of the linker, **L**, to the biologically active agent, **D**, producing a conjugate, **D-L**. The **D-L** construct is then reacted with multifunctional chemical moiety, **M**, producing the system **D-L-M**.

Reaction Scheme I

D	+	**L**	→	**D-L**
D-L	+	**M**	→	**D-L-M**
D-L-M	+	**P**	→	**poly[D-L-M-P]**

The **D-L-M** is then covalently coupled to the appropriate water soluble polymeric segment, **P**, to give the desired regular repeating linear polymeric drug conjugate **poly[D-L-M-P]**.

Assembly Method II

Alternatively, the linker, **L**, can first be coupled to **M**, to form **L-M**. (See Reaction Scheme 2.)

Reaction Scheme 2

L	+	**M**	→	**L-M**
L-M	+	**D**	→	**D-L-M**
D-L-M	+	**P**	→	**poly[D-L-M-P]**

The second reaction of this pathway requires the chemical coupling of **L-M** to agent, **D**, yielding the **D-L-M** which is then attached to the appropriate polymeric system, **P**, to form invented construct **poly[D-L-M-P]**.

Assembly Method III

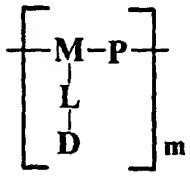
Alternatively, **M** can be initially coupled to the linker, **L**, (see Reaction Scheme 3) to prepare the conjugate **L-M**. **L-M** is then reacted with **P** and then **D** to prepare the invented construct.

Reaction Scheme 3

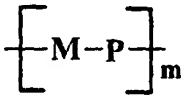
M	+	**L**	→	**L-M**
L-M	+	**P**	→	**poly[L-M-P]**
p ly[L-M-P]	+	**D**	→	**poly[D-L-M-P]**

See General Formula 4 for a description of structural abbreviations. Depending on the reaction conditions used in this synthetic pathway, the product constructs may or may not have each linker, L, substituted with a biologically active agent, D.

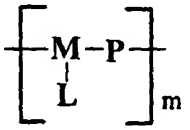
General Formula 4



= poly[D-L-M-P]



= poly[M-P]



= poly[L-M-P]

Assembly Method IV

Alternatively, **M**, can be covalently attached to the polymer, **P**, to yield the construct **poly[M-P]** (see Reaction Scheme 4). The co-polymer conjugate is then reacted with the linker, **L**, to yield **poly[L-M-P]** and is then coupled to the biologically active agent, **D**, to yield product **poly[D-L-M-P]**.

Reaction Scheme 4

M	+	**P**	→	**poly[M-P]**
poly[M-P]	+	**L**	→	**poly[L-M-P]**
poly[L-M-P]	+	**D**	→	**poly[D-L-M-P]**

Depending on the reaction conditions used in this synthetic pathway, the product constructs may or may not have each linker, **L**, substituted with a biologically active agent, **D**.

Assembly Method V

Alternatively, **M** can be reacted with **P** to form the polymer conjugate, **p ly[M-P]**, as shown in Reaction Scheme 5.

Reaction Scheme 5

M	+	**P**	→	**poly[M-P]**
D	+	**L**	→	**D-L**
poly[M-P]	+	**D-L**	→	**poly[D-L-M-P]**

The **D-L** can then be synthesized independently and attached to **poly[M-P]**, to yield the invented construct, **poly[D-L-M-P]**. Depending on the reaction conditions used in this synthetic pathway, the product constructs may or may not have each multifunctional moiety, **M**, substituted with a **D-L**.

Construct, $Q(-P-L-D)_k$

Assembly Method VI

The assembly of the branched polymeric drug conjugate $Q(-P-L-D)_k$ is illustrated in Reaction Scheme 6. An initial covalent coupling of the linker, **L**, to the biologically active agent, **D**, and the conjugation of the common multifunctional chemical moiety, **Q**, to two or more water soluble polymer segments, **P**, is followed by the attachment of both resultant conjugates to produce the invented construct.

Reaction Scheme 6

D	+	**L**	→	**D-L**
Q	+	**P**	→	**Q-P**
D-L	+	**Q-P**	→	$Q(-P-L-D)_k$

Depending on the reaction conditions used in this synthetic pathway, the product constructs may or may not have each polymer segment, **P**, substituted with **D-L**.

Assembly Method VII

Alternatively, **Q** can first be coupled to two or more polymer segments, **P**, to form $Q(-P)_k$. (See Reaction Scheme 7.)

Reaction Scheme 7

Q	+	**P**	→	**Q(-P)$_k$**
Q(-P)$_k$	+	**L**	→	**Q(-P-L)$_k$**
Q(-P-L)$_k$	+	**D**	→	**Q(-P-L-D)$_k$**

The second reaction of this pathway requires the chemical coupling of **Q-(P)$_k$** to the linker **L**, yielding the **Q-(P-L)$_k$** macromer which is then attached to the appropriate biologically active agent, **D**, to form invented construct **Q(-P-L-D)$_k$**. Depending on the reaction conditions used in this synthetic pathway, the product constructs may or may not have each linker, **L**, substituted with a biologically active agent, **D**.

Assembly Method XIII

Alternatively, the biologically active agent, **D**, can be initially coupled to the linker, **L**, (see Reaction Scheme 8) to prepare conjugate **L-D**. **L-D** is then reacted with **P** to produce the macromer **P-L-D**, which is in turn attached to the common multifunctional moiety, **Q**, to prepare the invented construct.

Reaction Scheme 8

D	+	**L**	→	**L-D**
L-D	+	**P**	→	**P-L-D**
P-L-D	+	**Q**	→	**Q(-P-L-D)$_k$**

Chemical Methods for the Assembly of the Polymeric Drug Conjugates, poly[D-L-M-P] and Q(-P-L-D)$_k$

The invented constructs are assembled by the covalent coupling of structural portions, **D**, **L**, **P** and **M** or with **D**, **L**, and with **Q**. These units are attached by means of the substituents X_1-X_6 which are chosen to allow for the formation of stable covalent bonds between the various units of the polymeric drug conjugates.

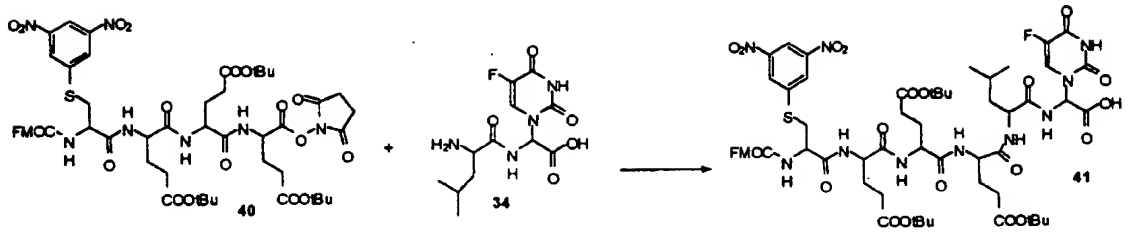
Attachment of the Pharmaceutically Active Agent, **D**, to the Linker, **L**

Many pharmaceutical agents, analogs, or metabolites, **D**, possess or can be modified to possess, reactive substituents for the formation of covalent bonds with linker **L**. Alternatively, a spacer group can be attached to **D** to allow for attachment to **L**. As discussed previously, theses substituents can independently be or be derived from, but are not restricted to, hydroxyl, amino, thiol, alkyl or aryl disulfide, isothiocyanate, thiocarbonylimidazole, thiocarbonylchloride, aldehyde, ketone, carboxylic acid, carboxylic acid ester, sulfonic acid, sulfonic acid ester, sulfonyl chloride, phosphoric acid, alkyl or aryl succinimidyl carbonate, alkyl or aryl chlorocarbonate, alkyl or aryl succinimidylthiocarbonate, alkyl or aryl chlorothiocarbonate, halide, or thioester functions (possibly substituted with appropriate protecting groups that can be removed before further chemical reaction). In some cases, additional reagents are added during the coupling reactions to begin or enhance the covalent attachments. The reagents and synthetic techniques needed for the coupling of **D** to **L** are commonly known to those of ordinary skill in the art of organic, peptide, or oligonucleotide synthesis. In cases when both functional groups X_5 and X_4 are present on **L** during a coupling reaction, X_4 is chemically protected to prevent reaction with **D**.

The preparation of the pharmaceutical agents and analogs is accomplished using techniques know by those of average experience in the art unless otherwise noted.

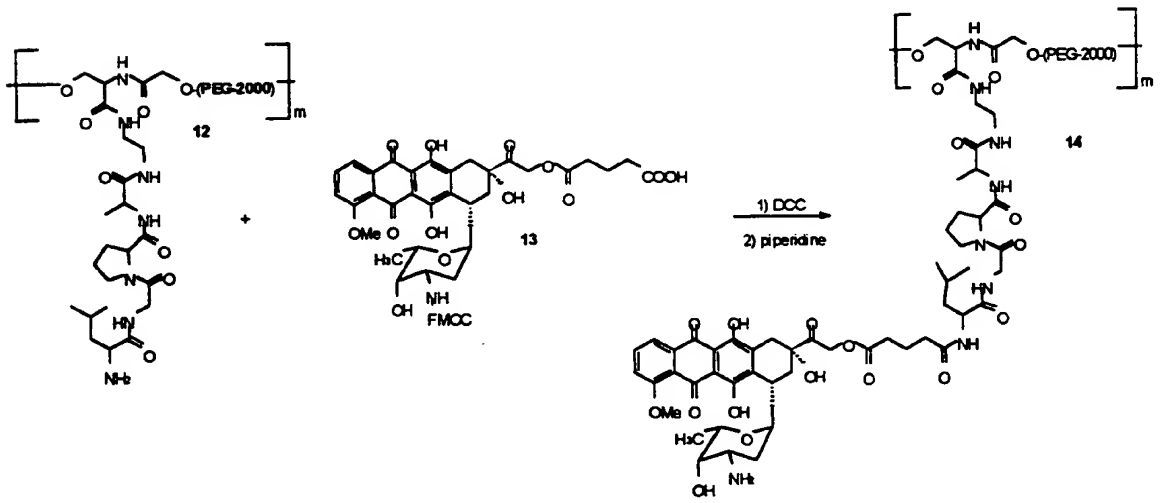
In an embodiment, functional group, X_5 on **L**, is the N-hydroxysuccinimidyl ester on tetrapeptide, Fmoc-Cys(Dnp)-Glu-Glu-Glu-OSu (Compound **40**) and the reactive substituent on **D** is the amino group on cancer drug analog H-Leu-Gly(α–5-fluorouracil)-OH (Compound **34**). The covalently coupled amide product **D-L** (Compound **41**) is shown in Reaction Scheme 9 below.

Reaction Scheme 9



In another embodiment, X_5 is the amino group on tetrapeptide conjugate, H-Leu-Gly-Pro-Ala-M-P (Compound **12**) and the reactive functionality on **D** is the carboxylic acid substituent of N-Fmoc-doxorubicin-14-O-hemiglutarate (Compound **13**). Activating agent 1,3-dicyclohexylcarbodiimide is added to the reaction mixture enabling the coupling reaction to take place. The covalently coupled amide product, regular repeating linear polymer **poly[D-L-M-P]**, (Compound **14**) is shown in Reaction Scheme 10 below.

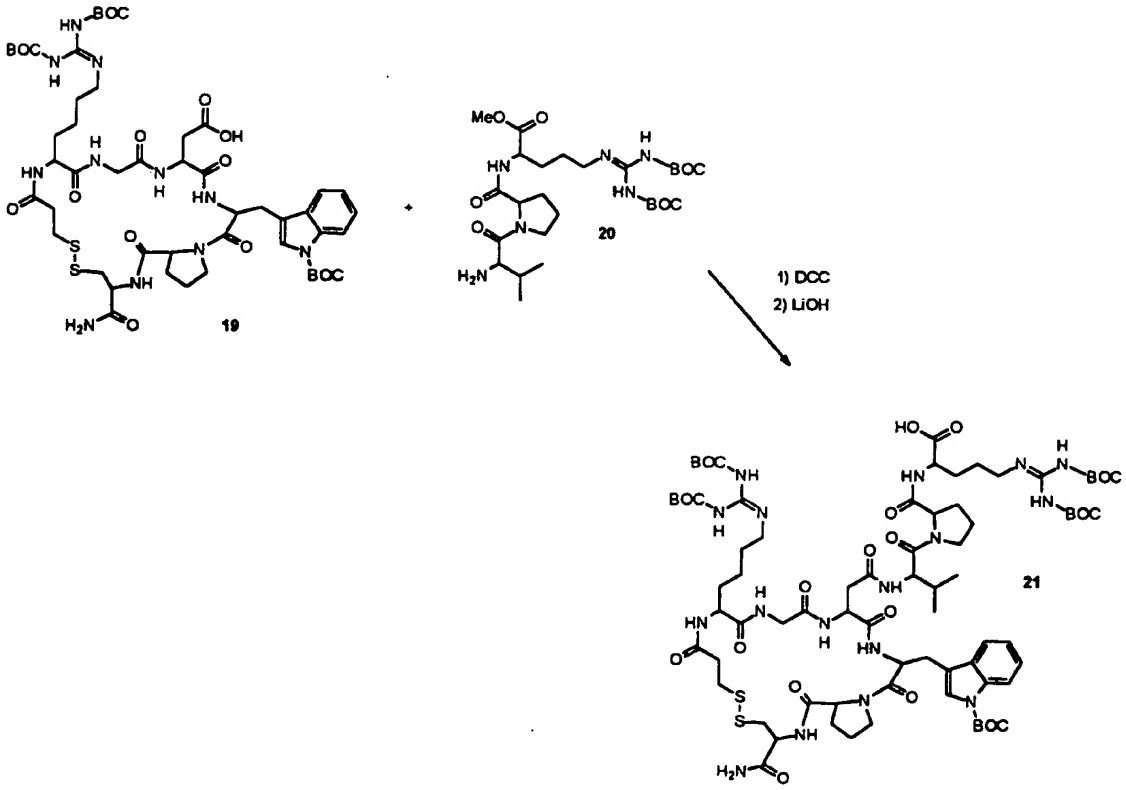
Reaction Scheme 10



In another embodiment, X_5 is the carboxylic acid function of an Integrilin analog

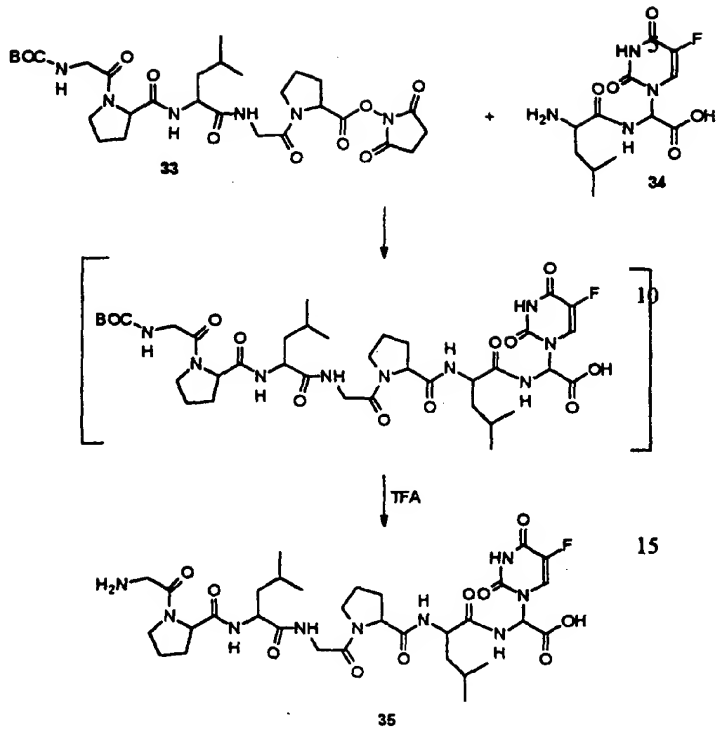
(Compound **19**) and the reactive function on **D** is the terminal amino group on tripeptide, H-Val-Pro-Arg(Boc)$_2$-OMe (Compound **20**). Activating agent 1,3-dicyclohexylcarbodiimide is added to the reaction mixture enabling the coupling reaction to take place. The covalently coupled amide product **D-L** (Compound **21**) is shown in Reaction Scheme 11 below.

Reaction Scheme 11



In another embodiment, functional group, X_5 on **L**, is the N-hydroxysuccinimidyl ester of pentapeptide, Boc-Gly-Pro-Leu-Gly-Pro-OSu (Compound **33**) and the reactive substituent on **D** is the amino group on cancer drug analog H-Leu-Gly(α-5-fluorouracil)-OH (Compound **34**). The covalently coupled amide product **D-L** (Compound **35**) is shown in Reaction Scheme 12 below.

Reaction Scheme 12



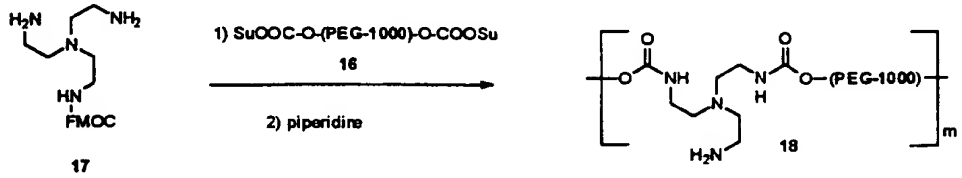
The Attachment of Water-Soluble Polymer Segment, **P** to the Multifunctional Chemical Moiety, **M**

The water soluble polymer segment, **P**, consists of a short, water soluble polymer or co-polymer system which contains at least two chemical functionalities for covalent attachment to the monomer, **M**. The reagents and synthetic techniques needed for the coupling of **M** to **P** are commonly known to those of ordinary skill in the art of organic, peptide, or oligonucleotide synthesis. **P** can be, but is not restricted to, polyethylene glycol, poly(vinyl alcohol), poly(2-hydroxyethyl methacrylate), poly(acrylic acid), poly(methacrylic acid), poly(maleic acid) or various copolymers or analogs of each. The preparation of the various polymers and analogs are accomplished using standard techniques known to those of average knowledge in the field of polymer synthesis. The attachment of common multifunctional moiety, **Q**, to polymer segment, **P**, is accomplished using synthetic reactions

similar to those for the conjugation of **M** to **P.**

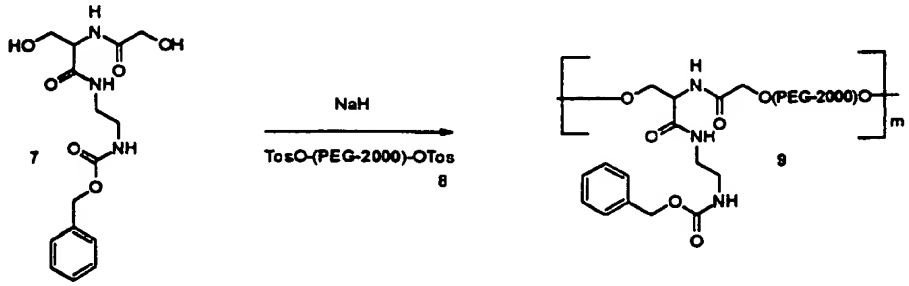
In one embodiment groups, X_1 and X_2 on , **M**, are both amino functions (Compound **17**) and the polymer, **P**, is an N-hydroxysuccinimidyl carbonate substituted analog of polyethylene glycol-1000 (Compound **16**). The resulting biscarbamate conjugate, regular repeating linear co-polymer, **P-M**, (Compound **18**) is shown in Reaction Scheme 13 below.

Reaction Scheme 13



In another embodiment, X_1 and X_2 are both hydroxy group (Compound **7**) and the reactive functionality on **P** (Compound **8**) is a toluene sulfonate ester. (See Reaction Scheme 14 below.) A base, sodium hydride, is added to the reaction mixture to initiate the covalent coupling and produce the ether product, regular repeating linear polymer, **poly[M-P]** (Compound **9**).

Reaction Scheme 14

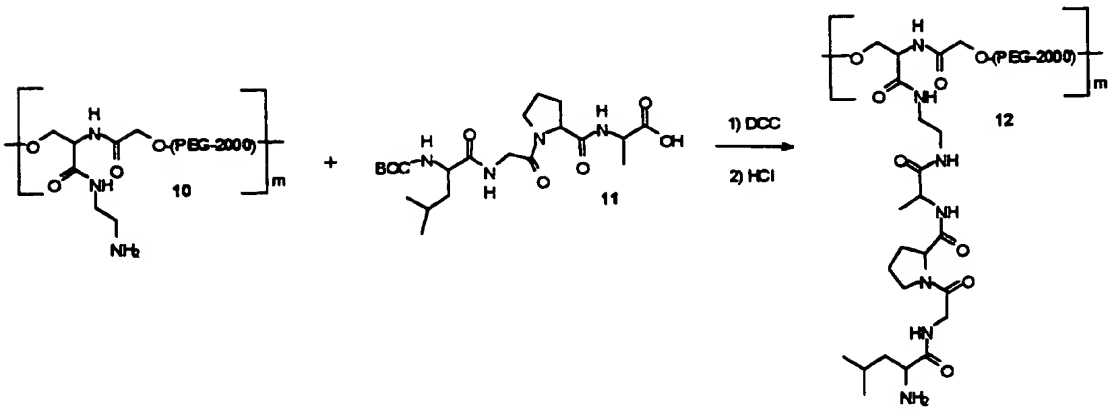


The Attachment of Linker, L to the Multifunctional Chemical Moiety, M

The coupling of the linker, L, to the multifunctional chemical moiety, M, is accomplished using the functions X_4 on L and X_3 on M. Substituents X_3 and X_4 can independently be or be derived from, but are not restricted to, hydroxyl, amino, thiol, alkyl or aryl disulfide, isothiocyanate, thiocarbonylimidazole, thiocarbonylchloride, aldehyde, ketone, carboxylic acid, carboxylic acid ester, sulfonic acid, sulfonic acid ester, sulfonyl chloride, phosphoric acid, alkyl or aryl succinimidyl carbonate, alkyl or aryl chlorocarbonate, alkyl or aryl succinimidylthiocarbonate, alkyl or aryl chlorothiocarbonate, halide, or thioester functions (possibly substituted with appropriate protecting groups that can be removed before further chemical reaction). The reagents and synthetic techniques needed for the coupling of L to M are commonly known to those of ordinary skill in the art of organic, peptide, or oligonucleotide synthesis.

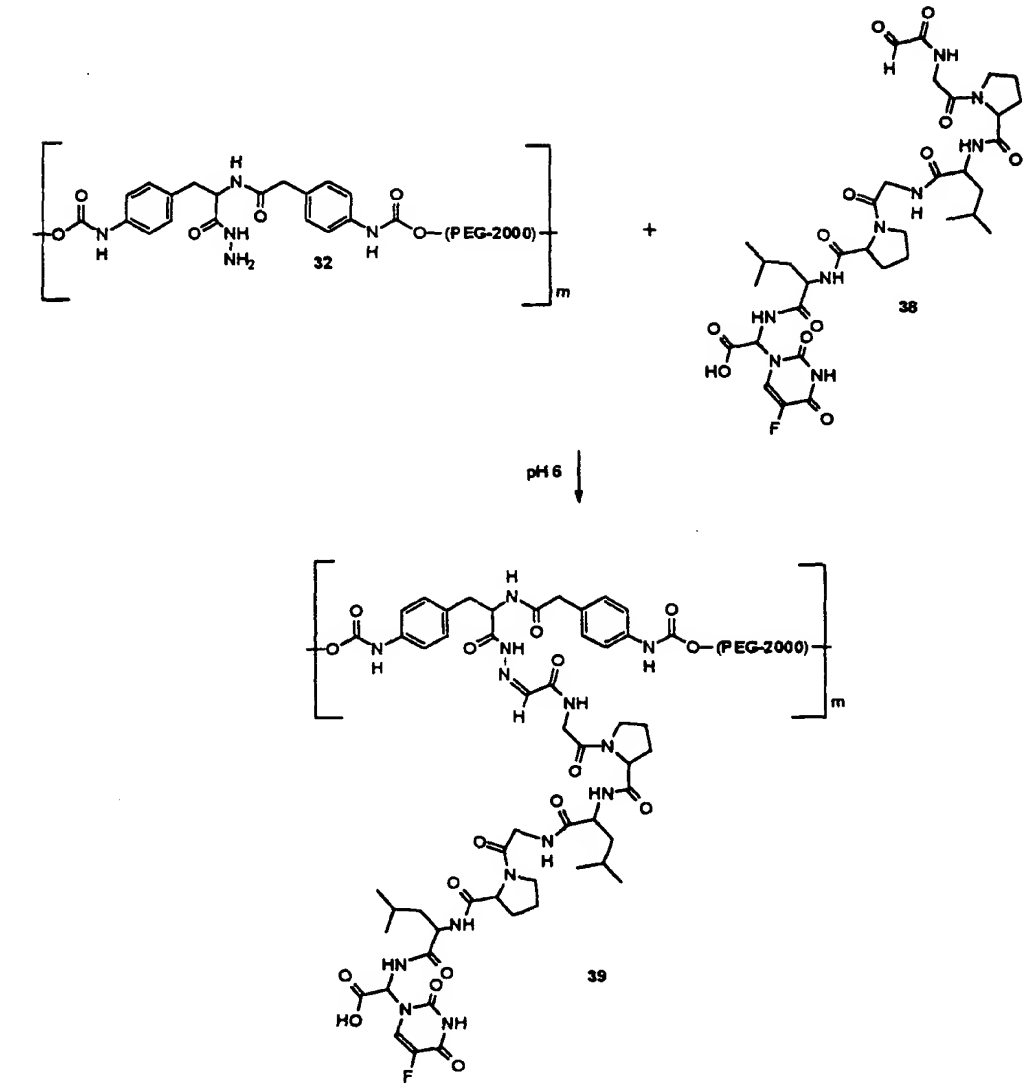
In one embodiment, group X_3 is an amine on M, already attached to the polymer system, P (Compound 10), and X_4 is a carboxylic acid on the linker, L (Compound 11), that is activated with 1,3-dicyclohexylcarbodiimide *in situ* to form the **poly[L-M-P]** portion of the invented construct. The structure of the resultant product (Compound 12) is shown in Reaction Scheme 15.

Reaction Scheme 15



In another embodiment, X_3 is an acid hydrazide on Compound **32** and X_4 is an aldehyde on Compound **38** (see Reaction Scheme 16).

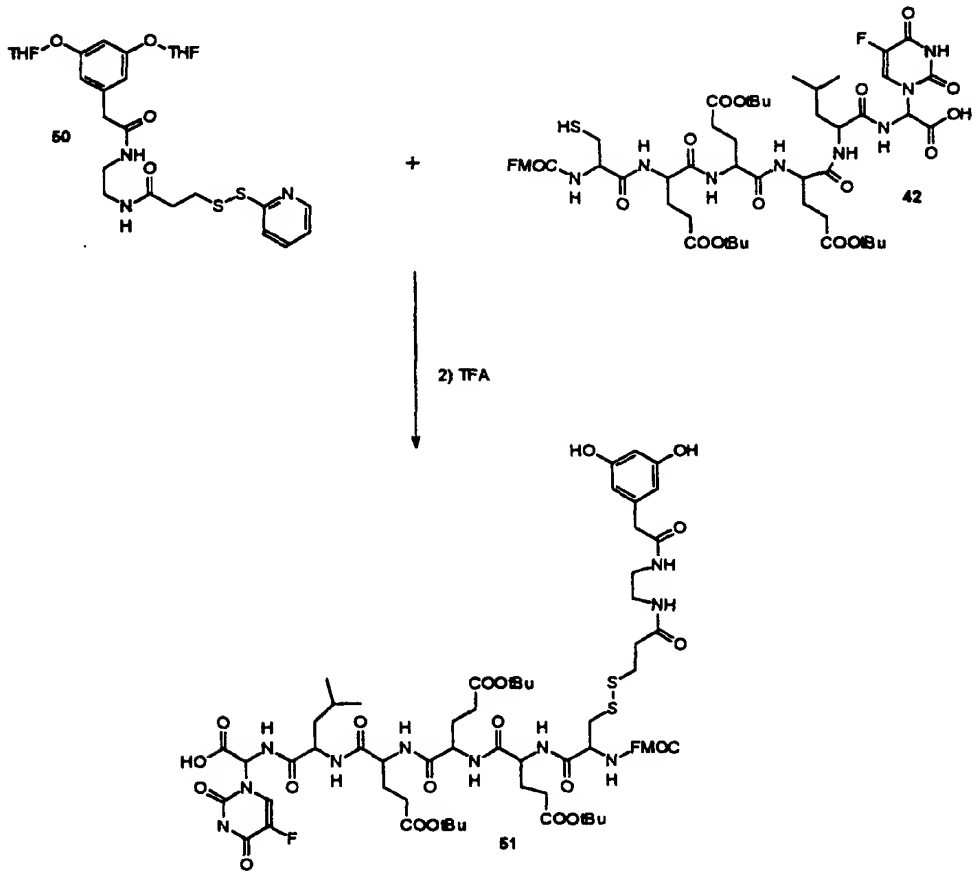
Reaction Scheme 16



5

In another embodiment, X_3 is a pyridyl disulfide on Compound **50** and X_4 is a thiol group on Compound **42** (see Reaction Scheme 17).

Reaction Scheme 17



Preparation of the Enzymatically Cleavable Linker, L

The linker, L, is assembled using standard synthesis methodologies and is designed to connect individual biologically active agent, D, to the polymeric scaffolds of the invented polymeric drug conjugates. It is also engineered to possess one or more enzymatically cleavable bonds, the breaking of which allows for the release of the biologically active agent or its analog from the polymeric constructs. The linker, L, may also include spacer groups, R_4 and R_5, that contain one or more hydrolytically, oxidatively, or photolytically cleavable chemical bonds. Since L contains two active functional group, X_4 and X_5, one or more of these functions can be chemically protected during the assembly of the construct and then deprotected when required. (A detailed list of chemical protecting groups and deprotection conditions can be found in Greene, et. al., "Protective Groups in Organic Synthesis," John Wiley & Sons, New York, 1981.)

The enzymatically cleavable bond of the linker may be spaced from the co-polymeric backbone and biologically active agent via the spacer groups to allow for enhanced exposure of the linking group to enzymes or to provide an optimal chemical environment for cleavage.

The assembly of L, which consists of independently or in combination, amino acids, sugars, nucleic acids, or other organic compounds joined by saturated and unsaturated, straight and branched chain alkyl, aryl, or alkylaryl, heteroalkyl, heteroaryl, or heteroalkyaryl groups which may contain up to 20 carbon atoms can be accomplished by using reagents and techniques known to those of average skill in the art of organic, peptide and oligonucleotide chemistry.

In a specific embodiment, the enzymatically cleavable linker is derived from the tripeptide, FMOC-Cys(DNP)-Glu(OtBu)-Glu(OtBu)-Glu(OtBu)-OSu which can be assembled using standard amino acid coupling techniques (see Lloyd-Williams, et al. "Chemical Approaches to the Synthesis of Peptides and Proteins," CRC Press, New York, 1997).

In another embodiment, the cleavable linker is derived from the tripeptide, H-Val-Pro-Arg(Boc)$_2$-OMe.

In another embodiment, the cleavable linker is derived from the tetrapeptide, H-Leu-Gly-Pro-Ala-OH.

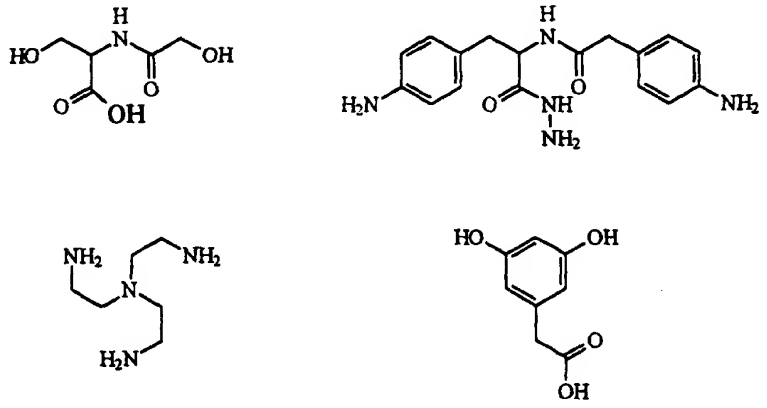
In another embodiment, the cleavable linker is derived from the pentapeptide, H-Gly-Pro-Leu-Gly-Pro-OSu.

Preparation of the Multifunctional Chemical Moiety, M

The multifunctional chemical moiety, **M**, is prepared using reagents and techniques known to one of average knowledge in the art. Since **M** contains three active functional group, X_1, X_2, and X_3, one or more of these functions can be chemically protected during synthesis and then deprotected when required. For a description of the many reactions which can be used to attach one portion of the construct to another see March, J.; Advanced Organic Chemistry, 4th Edition, John Wiley & Sons, New York, 1992. For a description of the reactions that can be used to prepared reactive functionalities on organic, peptide, and nucleotide moieties see Larock, R. C.: Comprehensive Transformations, VCH, New York, 1989, Bodansky, M.; Principles of Peptide Synthesis, Springer-Verlag, New York, 1984, and Mizuno, Y; The Organic Chemistry of Nucleic Acids, Elsevier, New York, 1986.

The structures of four multifunctional chemical moieties, **M**, or their precursors are shown below in Diagram 1.

Diagram 1

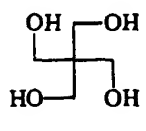


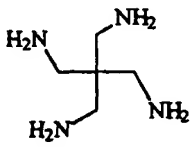
Preparation of the Common Multifunctional Chemical Moieties, Q

The multifunctional chemical moieties, **Q**, used to prepare the branched polymeric drug conjugates of the invention are prepared using reagents and techniques known to one of average knowledge in the art. Since **Q** contains multiple reactive functions X_6 that are designed to react with polymer segment **P** alone, no protecting groups are usually required. In some cases however, when more than one type of biologically active agent, **D**, enzymatically cleavable linker, **L**, or water soluble polymer segment, **P**, are desired on a single invented polymeric drug conjugate, some of the reactive functional groups may be protected during certain synthetic steps and then deprotected when required. For a description of the many reactions which can be used to attach one portion of the construct to another see March, J.; Advanced Organic Chemistry, 4th Edition, John Wiley & Sons, New York, 1992. For a description of the reactions that can be used to prepared reactive functionalities on organic, peptide, and nucleotide moieties see Larock, R. C.: Comprehensive Transformations, VCH, New York, 1989, Bodansky, M.; Principles of Peptide Synthesis, Springer-Verlag, New York, 1984, and Mizuno, Y; The Organic Chemistry of Nucleic Acids, Elsevier, New York, 1986.

The structures of two multifunctional monomer embodiments, **Q**, are illustrated below in Diagram 2.

Diagram 2





Mechanisms f Drug Release

The biologically active agent, **D**, is covalently coupled to the polymeric drug conjugates **poly[D-L-M-P]** and $Q(\text{-P-L-D})_k$ via a enzymatically cleavable bond present in the linker, **L**. The rate of release of **D** from either conjugate will depend on the mechanism of cleavage *in vivo*. **D** can be cleaved from the constructs by biological or physiological

processes, or by chemical reactions. **D** will be released either directly from the polymeric drug conjugate or in the form of a complex **D-L'** (a compound containing **D** coupled to all or part of **L**). The release of **D** may involve a combination of both enzymatic and non-enzymatic processes.

Cleavage (either by single or multiple steps) resulting in the release of the active agent **D** may be brought about by non-enzymatic processes. For example, chemical hydrolysis (for example, at an ester bond) may begin by simple hydration of the conjugate **poly[D-L-M-P]** upon delivery to the organism. Hydrolytic cleavage may result in the release of the complex **L-D** or the free active compound **D.** Cleavage can also be initiated by pH changes. For instance, the prodrug conjugate **poly[D-L-M-P]** may be dissolved in a minimally buffered acidic or basic pH solution before delivery; the bond between **L** and **D** or **M** and **L** of the prodrug conjugate **poly[D-L-M-P]** would be characterized by a high degree of chemical lability at physiological pH of 7.4, and would thus be cleaved when the conjugate is delivered to the tissue or circulatory system of the organism, releasing either the active agent **D** or the complex **L-D**. If necessary, a second reaction, either chemical or enzymatic, would result in the cleavage of **D** from the complex **L-D**. It is known to those in the art that N-Mannich base linkages exhibit this type of activity.

Cleavage can also occur due to an oxidative/reductive reaction. For example, a disulfide linkage can be created between **L** and **D** or **M** and **L** of the prodrug conjugate **poly[D-L-M-P]**. Such prodrug complexes would be stable at physiological pH; the bond between **L** and **D** or **M** and **L** of the prodrug conjugate **poly[D-L-M-P]** would be characterized by a high degree of chemical lability in reducing environments, such as in the presence of glutathione. If necessary, a second reaction, either chemical or enzymatic, would result in the cleavage of **D** from the complex **L-D**.

Proteolytic enzymes are produced in or near diseased tissues and organs as a result of biological signals from infectious agents, blood-borne cytokines, diseased tissues itself, or fluids near diseased tissue. In the present invention, the linking group **L** is designed to be cleaved between **L** and **D** or **M** and **L** of the conjugate **poly[D-L-M-P].** Such proteolytic enzymes can result from either the treated organism, or from microbial infection. Examples of such enzymes include, but are not restricted to: metalloproteinases and other extracellular matrix component proteases (including collagenases, stromelysins, matrilysin, gelatinases and

elastases), lysosomal enzymes (including cathepsin), serine proteases and other enzymes of the clotting cascade (such as thrombin), enzymes of the endoplasmic reticulum (such as cytochrome P450 enzymes, hydrolytic reaction enzymes and conjugation reaction enzymes), non-specific aminopeptidases and esterases, carboxypeptidases, phosphatases, glycolytic enzymes, and other enzymes that are present during certain disease conditions (such as angiotensin converting enzyme). Thrombin-like, alanine aminopeptidase, and elastase-like enzymatic activity are common in bacterial infections, and the amino acid cleavage sequences of such enzymes are well-documented.

There are many examples in the literature of possible amino acid sequences which can be used to cleave the linking group L at or near the site of diseased tissue in the constructs **poly[D-L-M-P]** and **Q(-G-L-D)$_k$**. For example, thrombin (a serine protease that is activated during the clotting cascade) cleaves the Arg-Gly bond in the following sequences:

-Gly-Arg-Gly-Asp-
-Gly-Gly-Arg-
-Gly-Arg-Gly-Asp-Asn-Pro-
-Gly-Arg-Gly-Asp-Ser-
-Gly-Arg-Gly-Asp-Ser-Pro-Lys-

Matrix metalloproteinases (MMPs) and other extracellular matrix proteases are prevalent in healing and metabolism. However, this enzyme family is also implicated in various pathological processes, including chronic inflammation, arthritis, and cancer. In particular, MMPs are active during tumor growth and are necessary for metastasis. One major extracellular protein is collagen, which has a characteristic repeat amino acid sequence: -Gly-Pro-Y-Gly-Pro-Z (where Y and Z are any amino acids, except for Pro or Hypro). Matrix metalloproteinases and other extracellular matrix proteases cleave primarily at Leu-Gly or Ile-Gly bonds. Amino acid sequences which are cleaved by this family of enzymes include, but are not restricted to:

-Gly-Pro-Leu-Gly-Pro-Z
-Gly-Pro-Ile-Gly-Pro-Z

-Pro-Leu-Gly-Pro-D-Arg-Z
-Ala-Pro-Gly-Leu-Z
-Pro-Leu-Gly-(Sleu)-Leu-Gly-Z
-Pro-Gln-Gly-Ile-Ala-Gly-Trp-
-Pro-Leu-Gly-Cys(Me)-His-
-Pro-Leu-Gly-Leu-Trp-Ala-

Other sequences which are cleaved by this family of enzymes include, but are not restricted to:

-Pro-Leu-Ala-Leu-Trp-Ala-Arg- (human Fibroblast Collagenase)
-Pro-Leu-Ala-Tyr-Trp-Ala-Arg- (human Neutrophil Collagenase)
-Pro-Tyr-Ala-Tyr-Trp-Met-Arg- (human Fibroblast Stromelysin)
-Pro-Leu-Gly-Met-Trp-Ser-Arg- (human Fibroblast or Neutrophil Gelatinases)
-Ala-Ala-Ala- (elastase)
-Ala-Ala-Pro-Ala- (elastase)
-Ala-Ala-Pro-Val- (elastase)
-Ala-Ala-Pro-Leu- (elastase)
-Ala-Ala-Pro-Phe- (elastase)
-Ala-Tyr-Leu-Val- (elastase)

Another example of an enzyme that is up-regulated due to disease, and therefore can be exploited in the current invention, is the angiotensin converting enzyme. This enzyme cleaves at amino acid sequences which include, but not restricted to:

-Asp-Lys-Pro-
-Gly-Asp-Lys-Pro-
-Gly-Ser-Asp-Lys-Pro-

Cells at the site of diseased tissue will produce numerous enzymes, growth factors, and cytokines that are present elsewhere in the organism at much lower concentrations. For

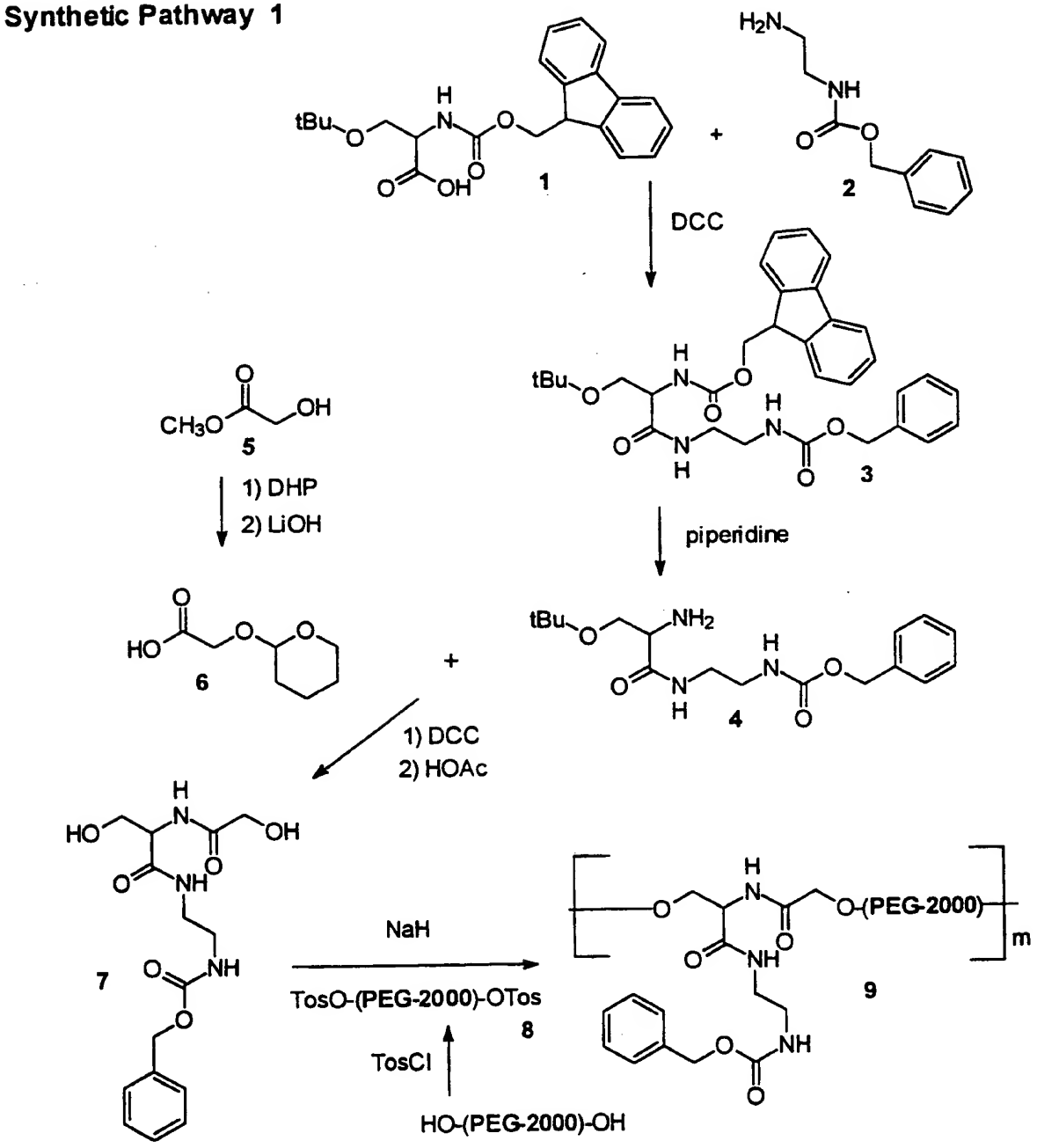
example, cells that are involved with inflammation that produce secreted and cell-surface enzymes include: granulocytes (neutrophils, eosinophils, basophils), monocytes/ macrophages, and lymphocytes. Activated macrophages are known to secrete elastase, collagenase and other MMPs, plasminogen activator, and other proteolytic enzymes. Activated peritoneal macrophages are known to produce hydrogen peroxide, which can be used to cleave **D** from the invented prodrug conjugates. Eosinophils, activated at the site of inflammation, produce lysosomal enzymes, peroxidase, histaminase, and other enzymes. As another example of disease-specific cleavage enzymes, various cancer cells (example, from prostate tumors) produce secreted or cell-surface enzymes that cleave specific amino acid sequences.

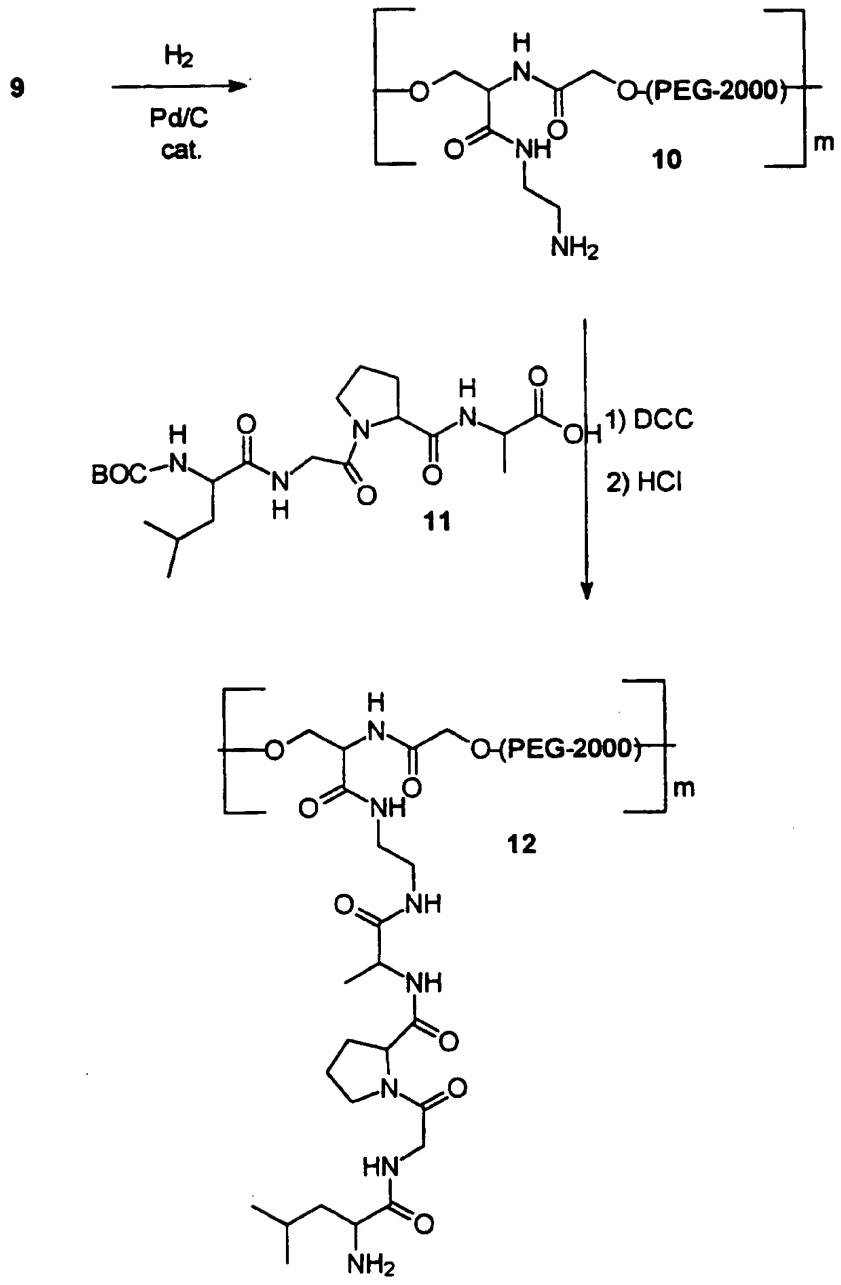
4. EXAMPLES

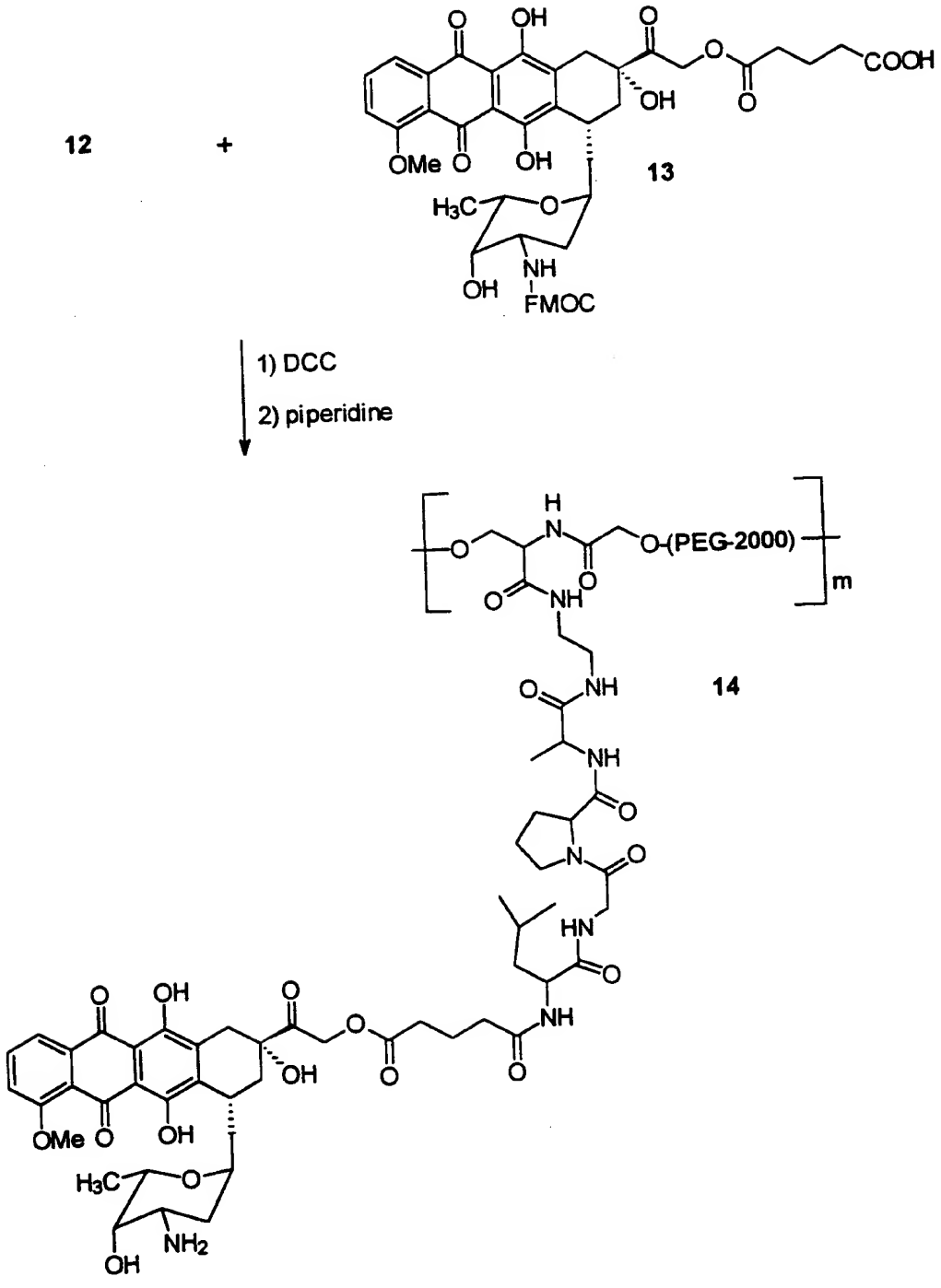
4.1 Assembly of Invented Drug Linked Polymer Conjugate, 14

The present example (see Synthetic Pathway 1) describes the preparation of a regular repeating linear polymeric drug conjugate of the invention in which the biologically active agent, **D**, is derived from Fmoc-doxorubicin-14-O-hemiglutarate, the enzymatically cleaved region of the linker, (L_1-L_n), is the tetrapeptide, Val-Gly-Pro-Ala, the multifunctional chemical moiety, **M**, is compound 7, and the water soluble polymer segment, **P**, is poly(ethylene glycol) with an average M.W. of about 2000 (**PEG-2000**).

Synthetic Pathway 1




9
H2
Pd/C
cat.
O-(PEG-2000)
m
10
NH2
BOC
1) DCC
2) HCl
11
12


12
+
13
1) DCC
2) piperidine
14
(PEG-2000)
m
COOH
OH
OMe
FMOC
NH2

Preparation of amide, **3**

A clean dry 500mL round bottom flask equipped with a magnetic stir bar, a thermometer, and a Drierite filled drying tube is charged with 200mL of dichloromethane, 3.83g (10mmol) of N-fluorenylmethoxycarbonyl-O-tert-butylserine, 1, (Bachem, King of Prussia, PA), 1.94g (10mmol) of N-(benzyloxycarbonyl)-ethane-1,2-diamine, 2 (prepared by the method of Denny et al., *Synthesis*, **1984**, 1032), and 2.06g (10mmol) of 1,3-dicyclohexylcarbodiimide (Aldrich, Milwaukee, WI). The resultant solution is magnetically stirred at room temperature for 5h and then 50mL of water is added. The reaction mixture is filtered, the aqueous layer is separated from the organic layer, and the organic layer is dried over anhydrous sodium sulfate for 4h. The slurry is filtered and the solvents are removed by rotary evaporation to leave a thick oil. Crystallization of the distillation residue using an appropriate solvent(s) affords the purified product amide, **3**.

Preparation of amine, **4**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 5.59g (10mmol) of **3**, 100mL of N,N-dimethylformamide, and 11mL of piperidine (Aldrich). The reaction mixture is stirred magnetically at room temperature for 1.0 h and then the solvents are removed by rotary evaporation. The distillation residue is triturated with 250mL of an appropriate solvent, the resultant precipitates are filtered, and the isolated solids are dried at reduced pressure to give amine, **4**.

Preparation of tetrahydropyranyl ether, **6**

A 250mL round bottom flask equipped with a thermometer, magnetic stir bar and Drierite filled drying tube is charged with 100mL of dichloromethane, 0.90g of methyl glycolate, **5**, (10mmol, Aldrich) 0.84g of 3,4-dihydro-2H-pyran (10mmol, Aldrich) and 0.1g of p-tolunesulfonic acid catalyst. The resultant solution is magnetically stirred at room temperature for 2h and then the solvents are removed by rotary evaporation. A solution consisting of 0.44g of lithium hydroxide monohydrate (11mmol) in 75mL of methanol and 25mL of water is added and the resultant slurry is stirred at 5 °C for 15h . An aqueous solution of 0.1N hydrochloric acid is slowly introduced until the reaction mixture is

neutralized, and 100mL of dichloromethane is added. The organic layer is separated from the aqueous layer, the organic layer is dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation to leave tetrahydropyranyl ether, **6**.

Preparation of amide, **7**

A clean dry 500mL round bottom flask equipped with a thermometer, Drierite filled drying tube and magnetic stir bar is charged with 200mL of dichloromethane, 1.60g (10mmol) of tetrahydropyaranyl ether, **6**, 3.37g (10mmol) of **4**, and 2.06g (10mmol) of 1,3-dicyclohexylcarbodiimide (Aldrich). The reaction mixture is magnetically stirred at room temperature for 5h and then 50mL of water is added. The slurry is filtered, the aqueous layer is separated from the organic layer and the organic layer is dried over anhydrous sodium sulfate for 4h. The resultant mixture is filtered, the solvents are removed by rotary evaporation, and 100mL of a solution of acetic acid:tetrahydro-furan:water/4:2:1 is added. The reaction mixture is heated to 45 ° C for 3.5h, the solvents are removed by rotary evaporation, and the distillation residue is crystallized using an appropriate solvent(s) to afford purified product amide, **7**.

Preparation of poly(ethylene glycol)-2000 di-4-toluenesulfonate (**DTS-PEG2000**), **8**

A clean dry 500mL round bottom flask equipped with a thermometer, addition funnel, magnetic stir bar, and dry N_2 inlet-outlet is charged with 3.0g (1.5mmol) of poly(ethylene glycol) avg. M.W. 2000 (Aldrich), 75mL of pyridine, and 1.14g (6.0 mmol) of toluenesulfonyl chloride. The reaction mixture is stirred magnetically at 0 deg. C for 12h and then poured onto 300mL of ice and water. The aqueous solution is extracted with 3x100mL of methylene chloride, the organic layers are combined, dried over anhydrous sodium sulfate for 4h, filtered and the solvents are removed by rotary evaporation. Crystallization of the distillation residue with an appropriate solvent(s)affords the purified polymeric product, **8**.

Preparation of polymer conjugate, **9**

A 500mL round bottom flask equipped with a thermometer, addition funnel, magnetic stir bar, and dry N_2 inlet-outlet is charged with 0.86g g (2.2mmol) of a 60% dispersion of sodium hydride in mineral oil. The dispersion is washed three times with 25mL of hexane

under a blanket of dry nitrogen and then 250mL of dry tetrahydrofuran (dried by refluxing over sodium benzophenone ketal) is added to the reaction flask. A solution consisting of 0.34g (1 mmol) of amide, **7** dissolved in 50mL of tetrahydrofuran is then added dropwise followed by 2.0g (1 mmol) of DTS-PEG2000, **8**, and 0.5g of 18-Crown-6 (Aldrich). The reaction mixture is heated to reflux, allowed to stir for 8h, at room temperature and then 0.1N HCl is slowly added to acidify the reaction mixture. The solvents are removed by rotary evaporation and the distillation residue is dissolved in 30mL of water. The aqueous solution is dialyzed against distilled water (Spectrapor membrane with molecular weight cutoff of 12,000-14,000) and the resultant solution is lyophilized to give polymer conjugate, **9**. Average molecular weight of the product is determined by GPC relative to PEG standards.

Preparation of polymer Conjugate, **10**

A 200mL hydrogenation flask is charged with 1.0g (0.5mmol) of conjugate **9**, 100mL of acetic acid, and 0.1g of 5% palladium-carbon catalyst. The reaction mixture is shaken and hydrogenated at 40psi of hydrogen for 16h at room temperature. The reaction solution is filtered through a pad of Celite and the solvents are removed by rotary evaporation to leave a viscous oil. The distillation residue is dissolved in 30mL of water and the aqueous solution is dialyzed against distilled water (Spectrapor membrane with molecular weight cutoff of 12,000-14,000). The resultant aqueous solution is lyophilized to give polymer conjugate, **10**.

Preparation of peptide substituted polymer conjugate, **12**

A 500mL round bottom flask equipped with a magnetic stir bar, thermometer, Drierite filled drying tube and addition funnel is charged with 1.0g (0.5mmol) of polymer conjugate, **10**, 200mL of dichloromethane, 0.23g (0.5mmol) of Boc-Leu-Gly-Pro-Ala-OH, **11**,(Bachem)and 0.11g (5mmol)of 1,3-dicyclohexylcarbodiimide (Aldrich). The reaction mixture is allowed to stir magnetically for 4h at room temperature, 50mL of water is then added, the organic layer is separated from the aqueous layer, and the aqueous layer is extracted with 3x100mL portions of dichloromethane. The organic layers are combined, washed with 1x100mL of saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. 25mL of 3N HCl and 50mL of ethyl acetate are added to the distillation residue and the resulting slurry

is stirred at room temperature for 0.5h. The solvents are removed by rotary evaporation, the residue is dissolved in 25mL of water, and the aqueous solution is dialyzed (Spectrapor membrane with molecular weight cutoff of 12,000-14,000) against distilled water. The solution is lyophilized to give the peptide substituted polymer conjugate, **12**. Amino acid analysis is performed to determine the amount of peptide, **11**, attached to **10** (by determining the ratio of leucine, glycine, proline, and alanine, to serine).

Preparation of invented drug linked polymer construct, **14**

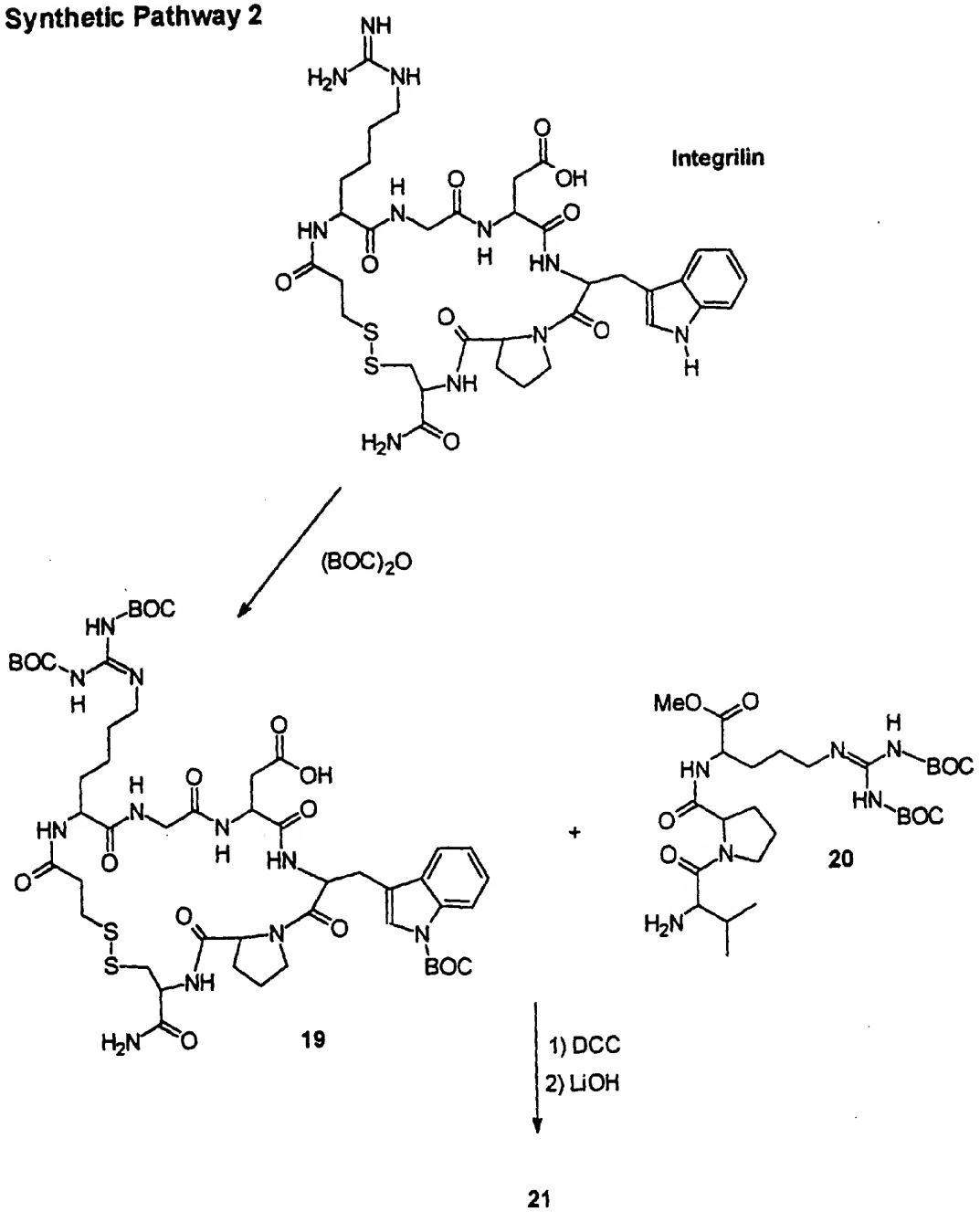
A 500mL round bottom flask equipped with a magnetic stir bar, thermometer, Drierite filled drying tube and addition funnel is charged with 0.5g (0.25mmol) of polymer conjugate, **12**, 200mL of dichloromethane, 0.40g (0.25mmol) of N-FMOC-doxorubicin-hemiglutarate (prepared by the method of Schally et al., Proc. Nat. Acad. Sci. USA, 1996, 93, 7269), and 0.06g (0.3 mmol) of 1,3-dicyclohexylcarbodiimide (Aldrich). The reaction mixture is allowed to stir magnetically for 4h at room temperature, 50mL of water is added, the organic layer is separated from the aqueous layer, and the aqueous layer is extracted with 3x100mL portions of dichloromethane. The organic layers are combined, washed with 1x100mL of saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. 1mL of piperidine in 5mL of N,N-dimethyl-formamide is added to the distillation residue and the resulting slurry is stirred at room temperature for 5min. The solvents are removed by rotary evaporation, the residue is dissolved in 25mL of water, and the aqueous solution is dialyzed (Spectrapor membrane with molecular weight cutoff of 12,000-14,000) against distilled water. The solution is lyophilized to give the invented drug linked polymer construct, **14**. The average molecular weight of **14** is determined by GPC analysis and the degree of substitution of **13** onto the product construct is calculated using UV and NMR spectroscopy.

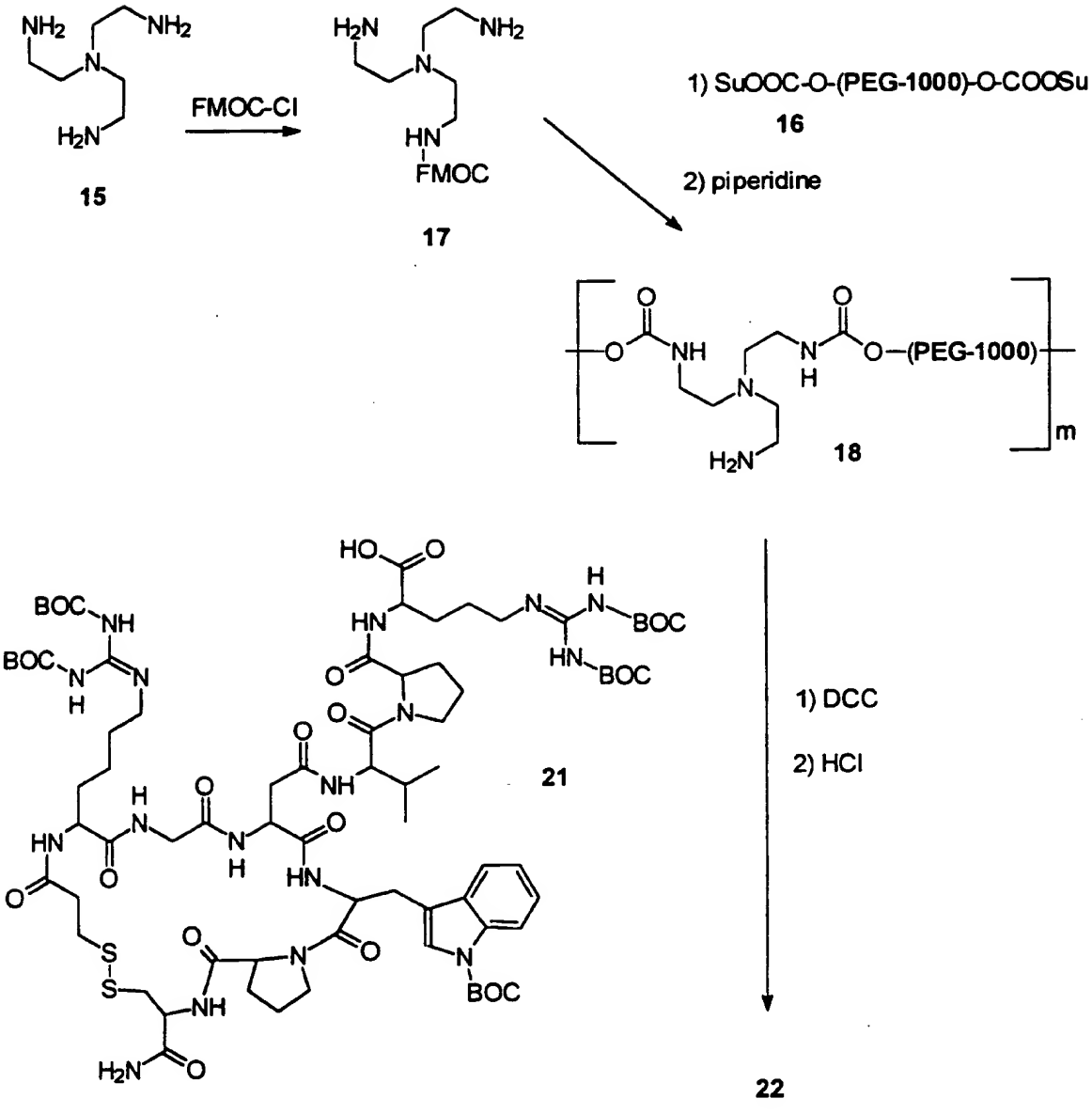
4.2 Assembly of Drug Linked Polymer Conjugate, 22

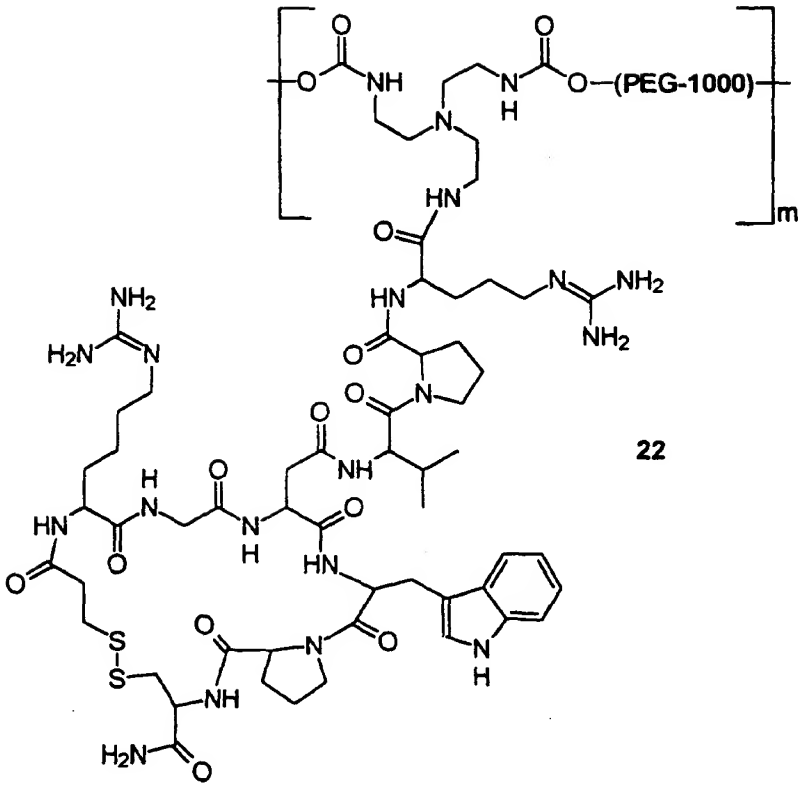
The present example describes the preparation (see Synthetic Pathway 2) of a regular repeating linear polymeric drug conjugate in which the pharmaceutical agent, **D**, is derived from Integrilin (N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic (1->6)-disulfide), the enzymatically

cleaved region of the linker, (L_1-L_n), is the tripeptide, Val-Pro-Arg, the multifunctional chemical moiety, **M**, is compound **17**, the water soluble polymer segment, **P**, is poly(ethylene glycol) with an average M.W. of about 1000 (**PEG-1000**).

Synthetic Pathway 2




FMOC-Cl
15
17
1) SuOOC-O-(PEG-1000)-O-COOSu
16
2) piperidine
18
m
21
1) DCC
2) HCl
22



Preparation of N-fluorenylmethoxycarbonyl-tris(2-aminoethyl)amine, 17

A 500mL round bottom flask equipped with a magnetic stir bar, thermometer, Drierite filled drying tube, and addition funnel is charged with 7.30g (50mmol) of tris(2-aminoethyl)amine, 15 (Aldrich) and 200mL of 1,4-dioxane. The reaction mixture is cooled to 5°C and then 2.60g (10mmol) of 9-fluorenylmethyl chlorocarbonate, FMOC-Cl (Aldrich), is slowly added. The reaction mixture is stirred for 12h at room temperature and then poured onto ice-water. The pH of the reaction mixture is adjusted to 8 using aqueous sodium carbonate solution and the aqueous solution is extracted with 3x100mL of dichloromethane. The organic layers are combined, washed with saturated sodium chloride solution, dried with anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to give purified N-fluorenylmethoxycarbonyl-tris(2-aminoethyl)amine, 17.

Preparation of polymer conjugate, **18**

A clean dry 500mL round bottom flask equipped with a magnetic stir bar, thermometer, Drierite filled drying tube, and addition funnel is charged with 0.37g (1mmol) of N-fluorenylmethoxy-carbonyl-tris(2-aminoethyl)amine, **17**, 5.0mL of 0.1M borate buffer at pH 9.3, and 1.14g (1mmol) of the bis-succinimidylcarbonate of poly(ethylene glycol) avg. M.W. 1000, **BSC-PEG-1000, 16**, (prepared using the procedure of Kohn et al., *Macromolecules*, **1992**, *25*, 4476). The reaction mixture is stirred for 15min and the solvents are removed by rotary evaporation. The distillation residue is stirred with 2mL of piperidine for 15min at room temperature and the solvents are removed by rotary evaporation. The distillation residue is mixed with 25mL of water, filtered and the filtrate is dialyzed (Spectrapor membrane with molecular weight cutoff of 12,000-14,000) against distilled water. The aqueous solution is lyophilized to afford the polymer conjugate product, **18**. GPC analysis against PEG standards is used to determine the molecular weight of the conjugate.

Preparation of tri-tert-butyloxycarbonyl protected Integrilin, **19**

A 250mL round bottom flask, equipped with a thermometer and magnetic stir bar is charged with 50mL of 1,4-dioxane, 25mL of water, 1mL of 1N sodium hydroxide, and 0.82g (1 mmol) of Integrilin (N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic (1->6)-disulfide). The reaction mixture is cooled to 10°C and 1.44g of di-tert-butyl dicarbonate (6.6 mmol; Aldrich) is added. The resultant solution is stirred at room temperature for 1h and the solvents are removed by rotary evaporation. The distillation residue is cooled in an ice-water bath and stirred in 50mL of ethyl acetate. A dilute solution of $KHSO_4$ is added slowly until a pH of 3 is attained and then the aqueous layer is extracted with 3x15mL of ethyl acetate. The organic layers are combined, washed with 2x50mL of water, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. Crystallization of the distillation residue using a suitable solvent(s) affords protected adduct, **19**.

Preparation of peptide conjugate, **21**

A 500mL round bottom flask equipped with a magnetic stir bar and Drierite filled

drying tube is charged with 200mL of dichloromethane, 1.12g (1 mmol) of Integrilin analog, **19**, 0.58g (1 mmol; Bachem) of H-Val-Pro-Arg(Boc)2-OMe, **20**, and 0.21g (1mmol) of 1,3-dicyclohexylcarbodiimide (Aldrich). The resultant solution is magnetically stirred at room temperature for 5h and then 50mL of water is added. The reaction mixture is filtered, the aqueous layer is separated from the organic layer, and the organic layer is dried over anhydrous sodium sulfate for 4h. The resultant slurry is filtered and the solvents removed by rotary evaporation to leave a syrup. The syrup is stirred with 1.1mL of 1N lithium hydroxide in 3mL of methanol at 5°C for 15h and then crystallized from an appropriate solvent to give peptide conjugate, **21**.

Preparation of invented drug linked polymer construct, **22**

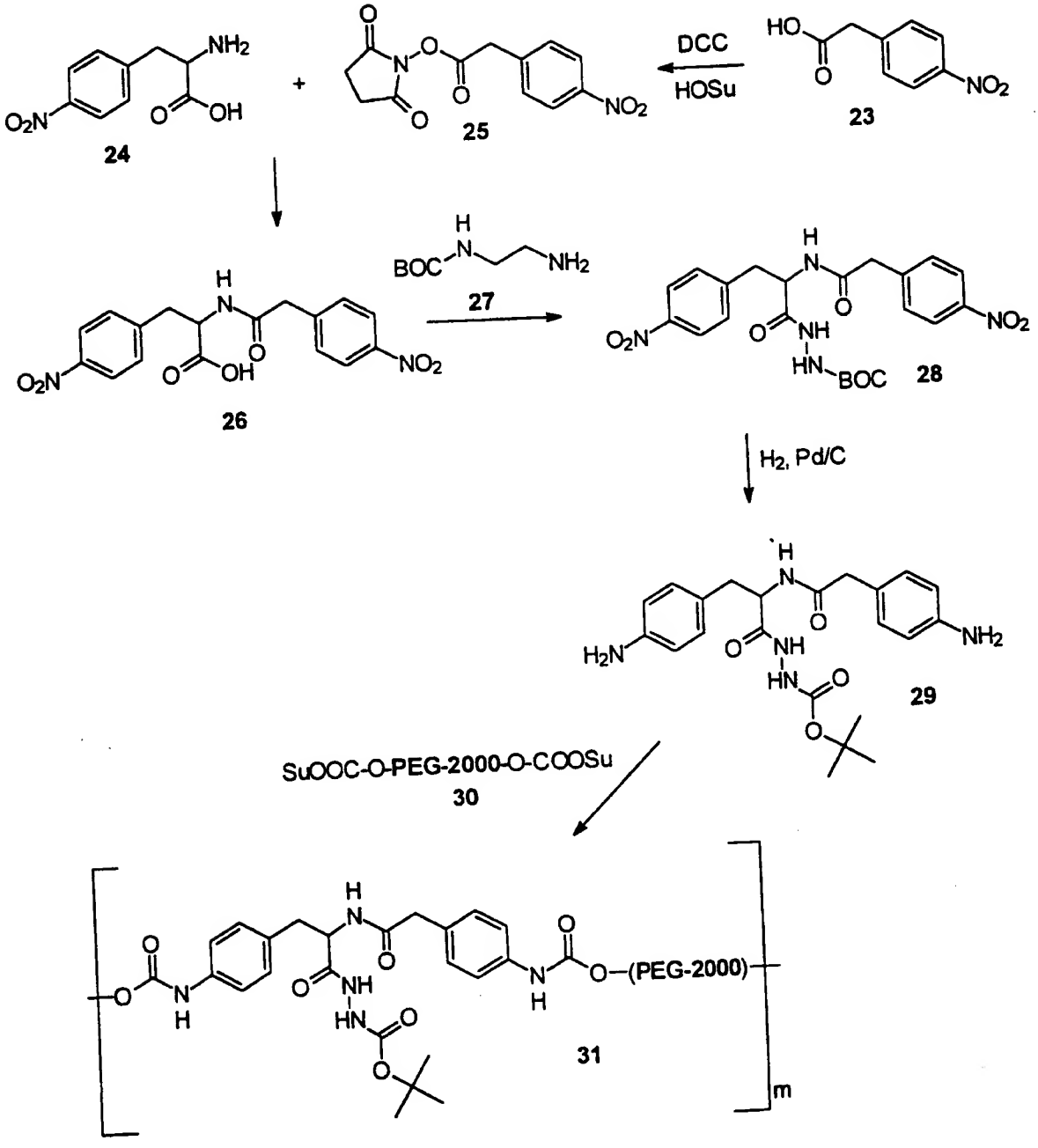
A 500mL round bottom flask equipped with a magnetic stir bar, thermometer, Drierite filled drying tube and addition funnel is charged with 0.5g (0.5mmol) of polymer conjugate, **18**, 200mL of dichloromethane, 0.84g (0.5mmol) of **21**, and 0.10g (0.3mmol) of 1,3-dicyclohexylcarbodiimide (Aldrich). The reaction mixture is allowed to stir magnetically for 4h at room temperature, 50mL of water is added, the organic layer is separated from the aqueous layer, and the aqueous layer is extracted with 3x100mL portions of dichloromethane. The organic layers are combined, washed with 1x100mL of saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. 25mL of 3N HCl and 75mL of ethyl acetate are added to the distillation residue and the resulting slurry is stirred at room temperature for 30min. The solvents are removed by rotary evaporation, the residue is dissolved in 25mL of water, and the aqueous solution is dialyzed (Spectrapor membrane with molecular weight cutoff of 12,000-14,000) against distilled water. The solution is lyophilized to give the invented drug linked polymer construct, **22**. The average molecular weight of **22** is determined by GPC analysis, the presence of the carbamate linkages is confirmed by IR spectroscopy, and the degree of substitution of **21** onto the product construct is calculated using UV and NMR spectroscopy.

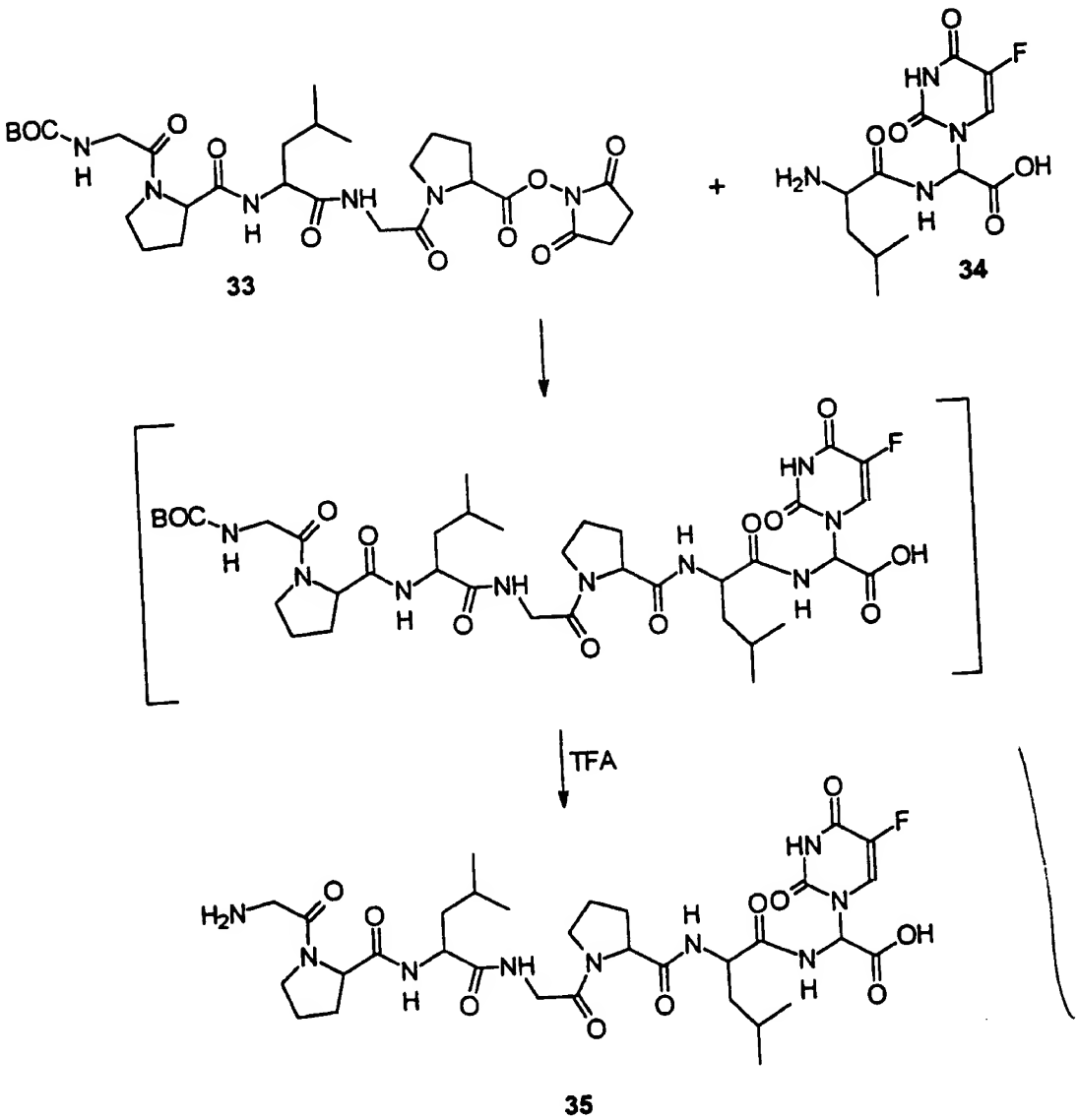
4.3 Assembly of Drug Linked P lymer Conjugate, 39

The present example describes the preparation (see Synthetic Pathway 3) of a regular

repeating linear polymeric drug conjugate of the invention in which the biologically active agent, **D**, is derived from H-Leu-Gly-α(5-fluorouracil)-OH, the enzymatically cleaved region of the linker, (L_1-L_n), is the heptapeptide, Gly-Pro-Leu-Gly-Pro-Leu-Gly, the multifunctional chemical moiety, **M**, is compound **29**, and the water soluble polymer segment, **P**, is poly(ethylene glycol) with an average M.W. of about2000 (**PEG-2000**).

Synthetic Pathway 3

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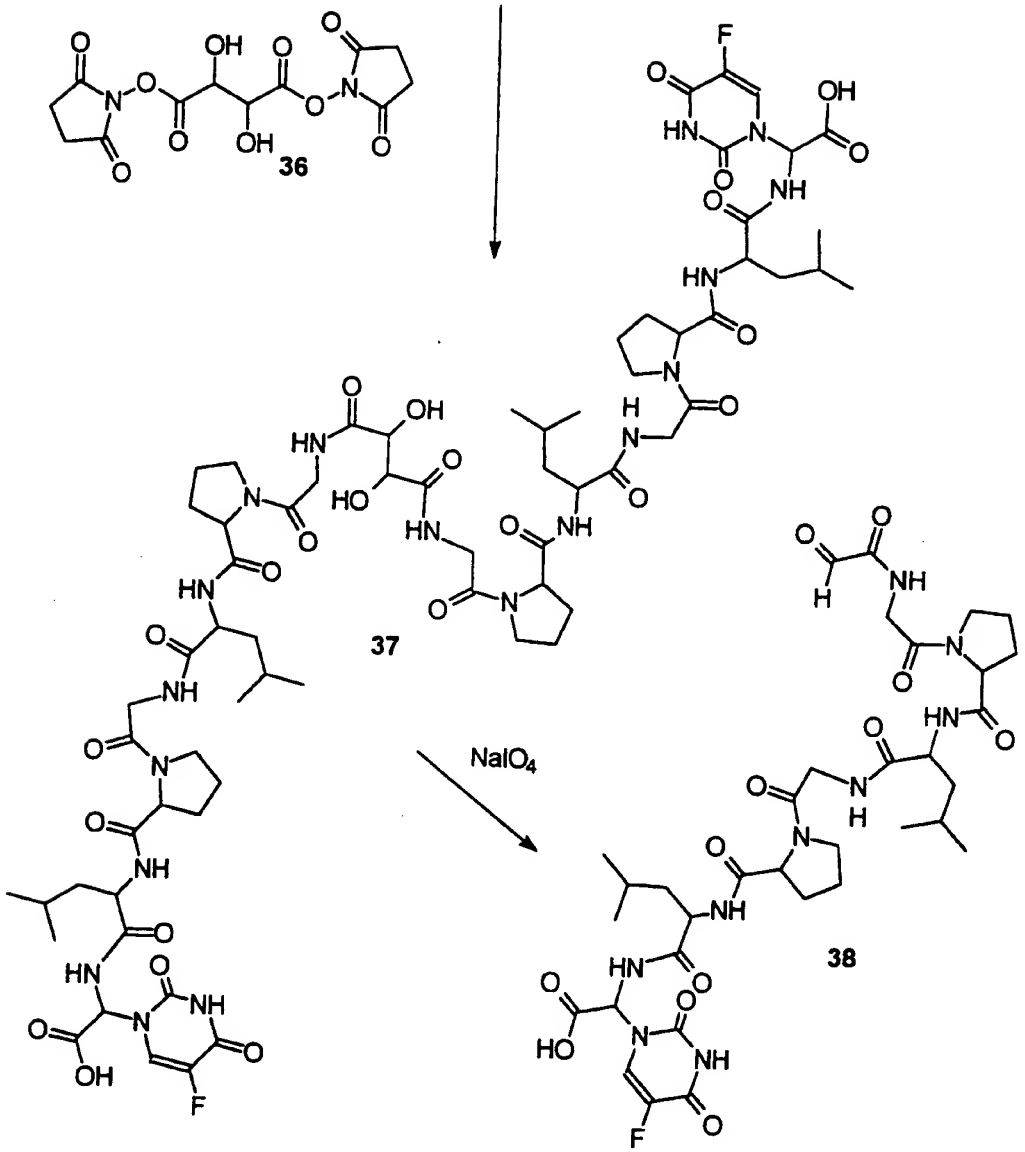
35



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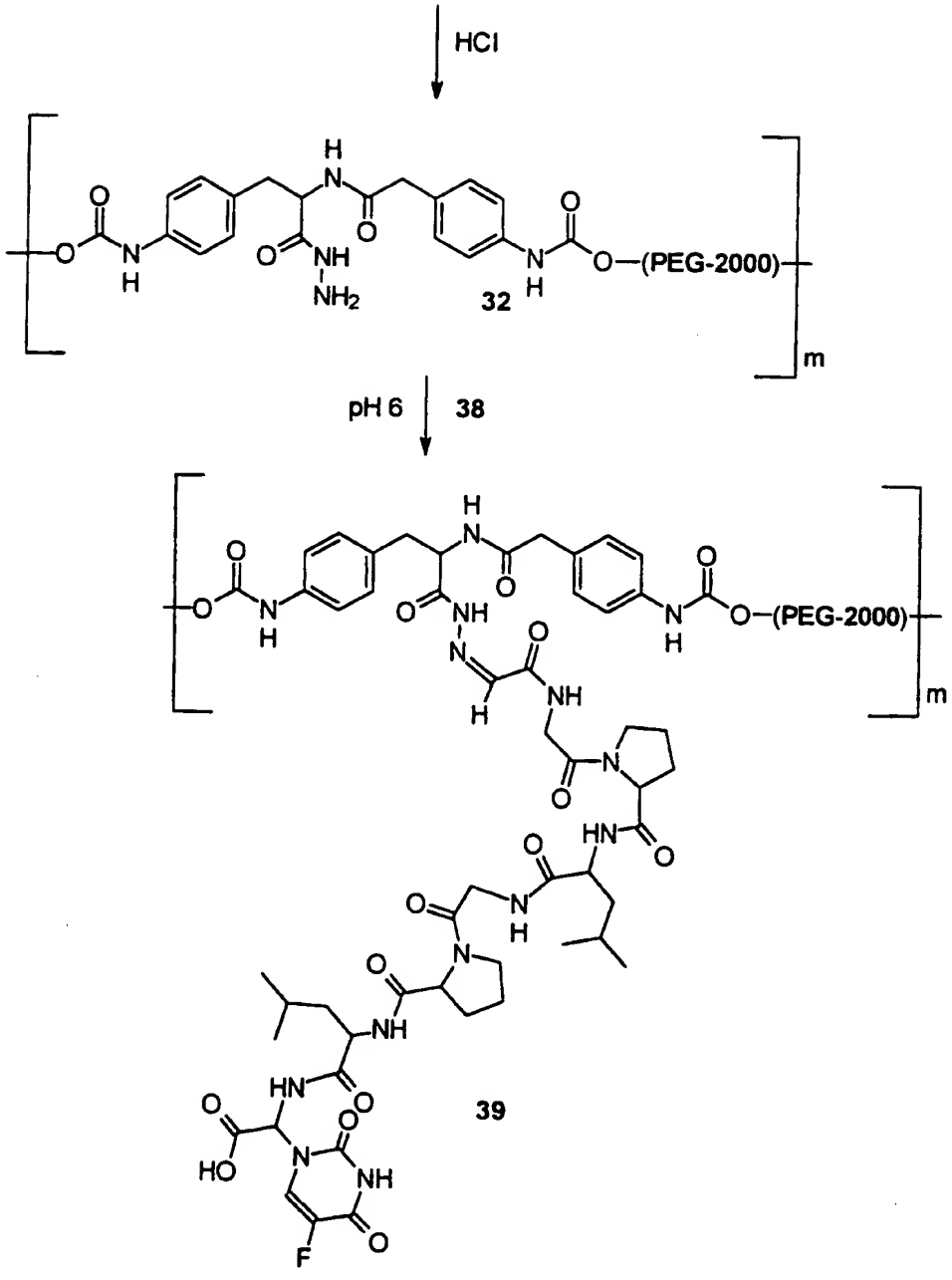


Preparation of p-nitrophenylacetic acid, N-hydroxysuccinimidyl ester, **25**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 1.81g (10mmol) of p-nitrophenylacetic acid (Aldrich), **23**, 200mL of 1,2-dimethoxyethane, 1.15g (10mmol) of N-hydroxysuccinimide (Aldrich), and 2.06g (10mmol) of 1,3-dicyclohexylcarbodiimide (Aldrich). The reaction mixture is stirred magnetically at room temperature for 12h, filtered, and the solvent is removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to give p-nitrophenylacetic acid, N-hydroxysuccinimidyl ester, **25**.

Preparation of amide, **26**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 2.78g (10mmol) of p-nitrophenylacetic acid, N-hydroxysuccinimidyl ester, **25**, 100mL of dry 1,2-dimethoxyethane, 1.40mL (10mmol) of triethylamine (Aldrich), and 2.10g (10mmol) of p-nitrophenylalanine (Schweizerhall, South Plainfield, NJ), **24**.The reaction mixture is stirred for 14 hour at room temperature and then poured onto a mixture 100mL of ice-water in 100mL of dichloromethane. The reaction mixture is filtered, the organic layer is separated from the aqueous layer, and the aqueous layer is extracted with 2x100mL of dichloromethane. The organic layers are combined, washed with 1x200mL of saturated sodium chloride solution, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to give amide, **26**.

Preparation of Boc protected hydrazide, **28**

A 500mL round bottom flask equipped with a magnetic stir bar and Drierite filled drying tube is charged with 200mL of dichloromethane, 3.73g (10mmol) of amide, **26**, 1.60g (10mmol; Aldrich) of N-tert-butyloxycarbonyl-1,2-diaminoethane (Aldrich), **27**, and 2.06g (1mmol) of 1,3-dicyclohexylcarbodiimide (Aldrich, Milwaukee, WI). The reaction mixture is magnetically stirred at room temperature for 5h and then 50mL of water is added. The reaction mixture is filtered, the aqueous layer is separated from the organic layer, and the

organic layer is dried over anhydrous sodium sulfate for 4h. The resultant slurry is filtered, the solvents removed by rotary evaporation, and the distillation residue is crystallized using an appropriate solvent(s) to give Boc protected hydrazide, **28**.

Preparation of diamine, **29**

A 200mL hydrogenation flask is charged with 4.87g (10mmol) of, 100mL of methanol, and 0.3g of 5% Pd-C catalyst (Aldrich). The resultant slurry is hydrogenated at 40psi of hydrogen for 16h at room temperature with mechanical shaking and then filtered through a pad of Celite. The solvents are removed from the filtrate by rotary evaporation and the distillation residue is crystallized using an appropriate solvent(s) to give diamine, **29**.

Preparation of 5-fluorouracil substituted peptide, **35**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 6.36g (10mmol) of Boc-Gly-Pro-Leu-Gly-Pro-OSu, **33** (Bachem), 3.16g (10mmol) of leucyl-2-(5-fluorouracil-1-yl)-L,D-glycine, **34** (prepared by the method of Putnam et al., *Bioconjugate Chem.*, **1995**, *6*, 483), 100mL of dry 1,2-dimethoxyethane, and 1.40mL (10mmol) of triethylamine (Aldich). The reaction mixture is stirred for 14 hour at room temperature, filtered, and the solvents are removed by rotary evaporation. The distillation residue is dissolved in a minimal amount of a mixture of acetonitrile in water and then purified using C-18 reverse phase HPLC (eluent: acetonitrile:water:0.1% trifluoroacetic acid). The collected eluent fractions are combined, and the solvents removed by rotary evaporation to give an oil that is dissolved in a minimal amount of distilled water. The aqueous solution is lyophilized to give the amine product, **35**.

Preparation of polymer conjugate, **31**

A clean dry 500mL round bottom flask equipped with a magnetic stir bar, thermometer, Drierite filled drying tube, and addition funnel is charged with 4.27g (10mmol) of diamine, **29**, 5 mL of N,N-dimethylformamide, 5.0mL of 0.1M borate buffer at pH 9.3, and 22.80g (10mmol) of the bis-succinimidylcarbonate of poly(ethylene glycol) avg. M.W. 2000, **BSC-PEG-2000, 30** (prepared using the procedure of Kohn et al., *Macromolecules*, **1992**, *25*, 4476). The reaction mixture is stirred for 15min and the solvents are removed by rotary

evaporation. The distillation residue is mixed with 25mL of water, filtered and the filtrate is dialyzed (Spectrapor membrane with molecular weight cutoff of 12,000-14,000) against distilled water. The aqueous solution is lyophilized to afford the polymer conjugate product, **31**. GPC analysis against PEG standards is used to determine the molecular weight of the conjugate.

Preparation of peptide adduct, **37**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 3.44g (10mmol) of disuccinimidyl tartarate, **36** (Pierce), 100mL of dry 1,2-dimethoxyethane, 1.40mL (10mmol) of triethylamine (Aldrich), and 7.37g (10mmol) of **35**. The reaction mixture is stirred for 14 hour at room temperature, and then poured onto 100mL of ice-water in 100mL of dichloromethane. The reaction mixture is filtered, the organic layer is separated from the aqueous layer, and the aqueous layer is extracted with 2x100mL of dichloromethane. The organic layers are combined, washed with 1x200mL of saturated sodium chloride solution, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to afford peptide adduct, **37**.

Preparation of peptidoaldehyde, **38**

A clean 250mL round bottom flask equipped with a thermometer and magnetic stirrer is charged with 1.59g (2mmol) of peptide adduct, **37**, and 100mL of 0.015M aqueous sodium periodate. The pH of the reaction mixture is adjusted to 7.0 using sodium phosphate buffer and the reaction solution is allowed to stir for 2h at room temperature. The solvents are removed by rotary evaporation and the distillation residue is dissolved in a minimal amount of a 1:1 mixture of water and acetonitrile. The resultant solution is injected onto a semi-preparative C-18 HPLC column and the product is eluted using a water:acetonitrile:0.1%TFA gradient. The collected fractions are combined and lyophilized to give peptidoaldehyde, **38**.

Preparation of acid hydrazide substituted polymer conjugate, **32**

A clean 250mL round bottom flask is charged with 1.0g of polymer conjugate, **31**, 25mL of water and 5mL of trifluoroacetic acid. The reaction mixture is shaken and the

solvents are removed by rotary evaporation. The distillation residue is dissolved in a minimal amount of water, filtered, and dialyzed (Spectrapor membrane with molecular weight cutoff of 12,000-14,000) against distilled water. The aqueous solution is lyophilized to afford acid hydrazide substituted polymer conjugate, **32**.

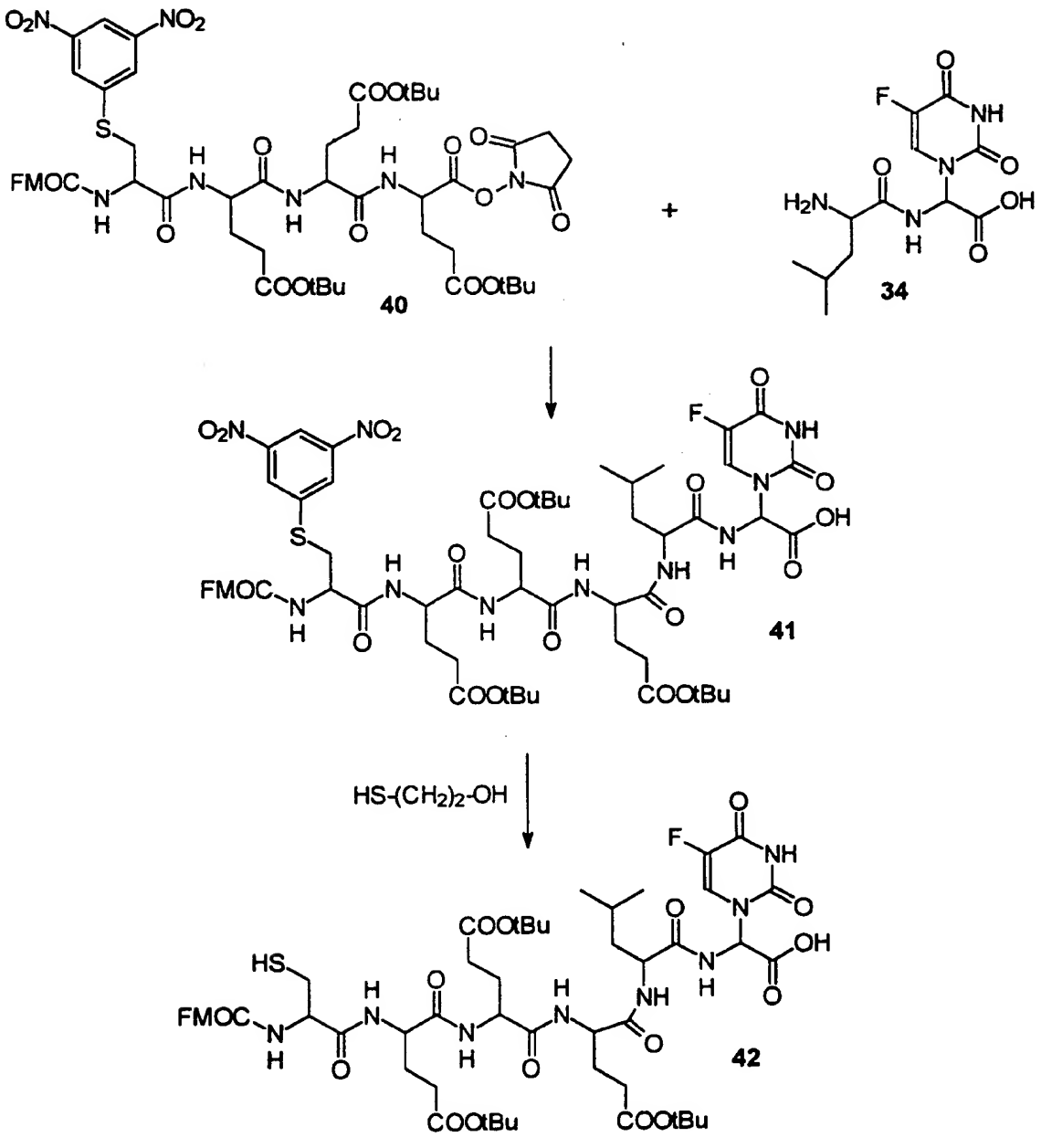
Preparation of invented drug linked polymer conjugate, **39**

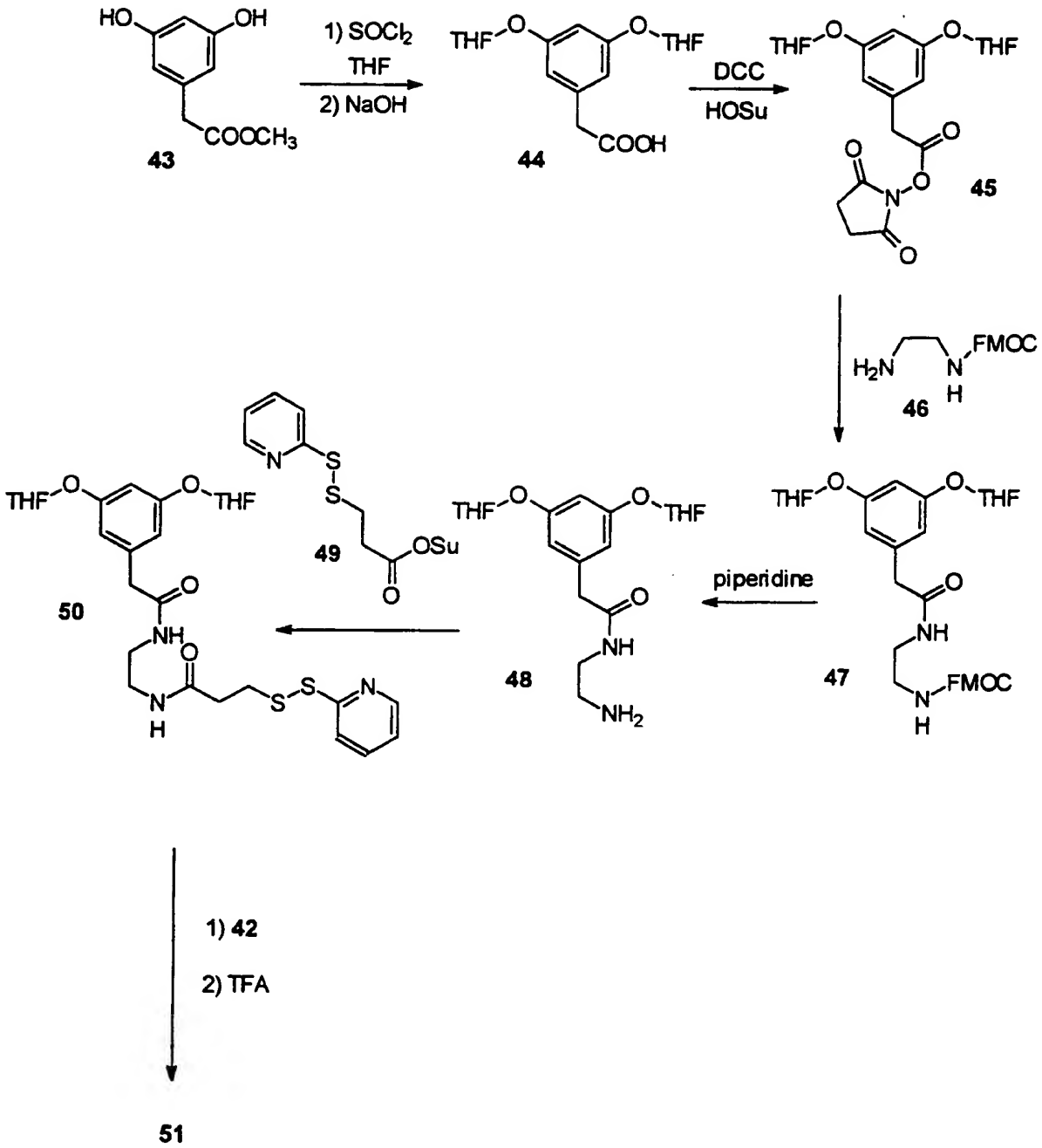
A clean 250mL round bottom flask equipped with a thermometer and magnetic stir bar is charged with 50mL of phosphate buffered saline solution at pH 5.0, 1.0g (0.5mmol) of acid hydrazide substituted polymer conjugate, **32**, and 0.40g (0.5mmol) of peptidoaldehyde, **38**. The reaction mixture is stirred for 20h at room temperature and then dialyzed against distilled water. The aqueous solution is lyophilized to give the invented drug linked polymer conjugate, **39**. The average molecular weight of **39** is determined by GPC analysis, the presence of the carbamate linkages is confirmed by IR spectroscopy, and the degree of substitution of **38** onto the product construct is calculated using UV and NMR spectroscopy.

4.4 Assembly of Drug Linked Polymer Conjugate, 53

The present example describes the preparation (see Synthetic Pathway 4) of a regular repeating linear polymeric drug conjugate of the invention in which the pharmaceutical agent, **D**, is derived from H-Leu-Gly-α(5-fluorouracil)-OH; the enzymatically cleaved region of the linker, (L_1-L_n), is hexapeptide, Cys-Glu-Glu-Glu-Leu-Gly; the multifunctional chemical moiety, **M**, is compound **51**: and the water soluble polymer segment, **P**, is poly(ethylene glycol) (**PEG-1000**) with an average M.W. of about 1000.

Synthetic Pathway 4

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THF
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THF
O
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COOH
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DCC
HOSu
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N
50
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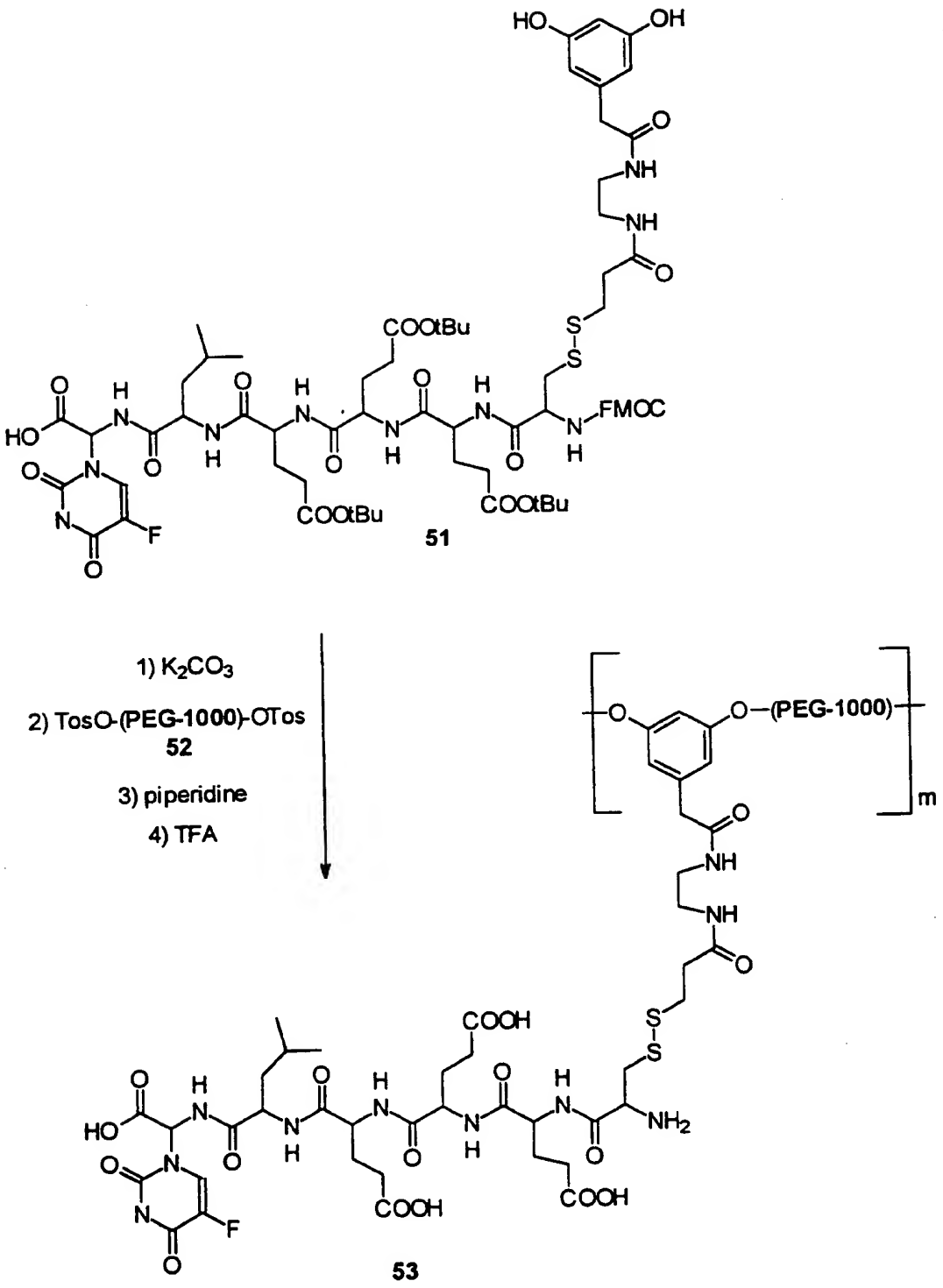
2) TFA
51



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53

Preparation of FMOC-L-Cys(DNP)-L-Glu(OtBu)-L-Glu(OtBu)-L-Glu(OtBu)-L-Leu-2-(5-fluorouracil-1-yl)-L,D-gly-OH, **41**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 11.62g (10mmol) of Fmoc-Cys(2,4-dintirophenyl(DNP))-Glu(OtBu)-Glu(OtBu)-Glu(OtBu)-Osu, **40** (Bachem), 100mL of dry 1,2-dimethoxyethane, 1.40mL (10mmol) of triethylamine (Aldrich), and 3.16g (10mmol) leucyl-2-(5-fluorouracil-1-yl)-L,D-glycine, **34** (prepared by the method of Putnam et al., *Bioconjugate Chem.*, **1995**, 6, 483). The reaction mixture is stirred for one hour at room temperature and then poured into a 400mL of ice-cold water. A sufficient amount of 0.1N HCl is slowly added to bring the pH of the solution to pH 5, the precipitates are filtered, dried, and dissolved in a minimal amount of a mixture of acetonitrile and water. The resultant solution is chromatographed using C-18 reverse phase HPLC (eluent: acetonitrile:water:0.1% trifluoroacetic acid), the collected eluent fractions are combined, and the solvents removed by rotary evaporation to give Fmoc-L-Cys(DNP)-L-Glu(OtBu)-L-Glu(OtBu)-L-Glu(OtBu)-L-Leu-2-(5-fluorouracil-1-yl)-L,D-gly-OH, **41**.

Preparation of Fmoc-L-Cys-L-Glu(OtBu)-L-Glu(OtBu)-L-Glu(OtBu)-L-Leu-2-(5-fluorouracil-1-yl)-L,D-Gly-OH, **42**

A clean 250mL round bottom flask equipped with a magnetic stir bar and thermometer is charged with 6.82g (5mmol) of Fmoc-L-Cys(DNP)-L-Glu(OtBu)-L-Glu(OtBu)-L-Glu(OtBu)-L-Leu-2-(5-fluorouracil-1-yl)-L,D-Gly-OH, **41**, and 50mL of 2-mercaptoethanol (Aldrich). 1N sodium hydroxide solution is added until a pH of 8 is attained and then the reaction mixture is stirred at room temperature for 1h. The solvents are removed by rotary evaporation and the distillation residue is crystallized using an appropriate solvent to give Fmoc-L-Cys-L-Glu(OtBu)-L-Glu(OtBu)-L-Glu(OtBu)-L-Leu-2-(5-fluorouracil-1-yl)-L,D-Gly-OH, **42**.

Preparation of 3,5-(2-ditetrahydrofuranyloxyxy)-phenylacetic acid, **44**

A clean 250mL round bottom flask equipped with a magnetic stir bar, dry nitrogen gas inlet-outlet, and thermometer is charged with 48mL of dry tetrahydrofuran and then 2.16g (16mmol) of sulfuryl chloride (Aldrich) is slowly added. The reaction mixture is stirred at

room temperature for 15min and simultaneously added with a solution of triethylamine (3.64g, 36mmol) in tetrahydrofuran (5mL) to a stirred solution of 0.91g (5mmol) of 3,5-dihydroxyphenylacetic acid, **43** (Aldrich), in 25mL of tetrahydrofuran over 0.5h. The reaction mixture is stirred for a further 1h at 40 ° C, cooled to –16°C, filtered, the solvents are removed from the filtrate by rotary evaporation. The distillation residue is slurried with 2x30mL of diethyl ether, the supernatants are combined, dried over anhydrous sodium sulfate, filtered, and the solvents are removed by rotary evaporation. The distillation residue is dissolved in a solution consisting of 5mL of 0.8M aqueous sodium hydroxide dissolved in 30mL of methanol and stirred at room temperature for 15min. The pH of the reaction mixture is adjusted to 6 by adding 1.0N HCl and then 100mL of ethyl acetate is added. The organic layer is separated from the aqueous layer, the organic layer is dried over anhydrous sodium sulfate for 4h, filtered and the solvents are removed by rotary evaporation to afford 3,5-(2-ditetrahydrofuranyloxyxy)-phenylacetic acid.

Preparation 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-hydroxy succinimidyl ester, **45**

A clean, dry, 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 3.08g (10mmol) of 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, **44**, 200mL of 1,2-dimethoxyethane, 1.15g (10mmol) of N-hydroxysuccinimide (Aldrich), and 2.06g of 1,3-dicyclohexylcarbodiimide (Aldrich). The reaction mixture is stirred magnetically at room temperature for 12h, filtered, and the solvents are removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to give 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-hydroxy succinimidyl ester, **45**.

Preparation of 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-(N'-fluorenyl-methoxycarbonylethyanediamine amide, **47**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 4.05g (10mmol) of 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-hydroxy succinimidyl ester, **45**, 100mL of dry 1,2-dimethoxyethane, 1.40mL (10mmol) of triethylamine (Aldrich), and 2.82g (10mmol) of N-

fluorenylmethoxycarbonylethanediamine trifluoroacetate, **46** (prepared according to the procedure of Adamczyk, et al., *Organic Preparations and Procedures International*, **1995**, *27*, 239).The reaction mixture is stirred for 14 hours at room temperature and then 50mL of water and 100mL of dichloromethane is added. The organic layer is separated from the aqueous solution and the aqueous layer is washed with 2x100mL of dichloromethane. The organic layers are combined, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to give 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-(N'-fluorenylmethoxy-carbonyl) ethanediamine amide, **47**.

Preparation of 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-ethanediamine amide, **48**

A clean dry, 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 5.72g (10mmol) of 3,5-(2-ditetrahydro-furanyloxy)-phenylacetic acid, N-(N'-fluorenylmethoxycarbonyl) ethanediamine amide, **47**, 100mL of N,N-dimethylformamide, and 11mL of piperidine (Aldrich). The reaction mixture is stirred magnetically at room temperature for 1.5h and the solvents are removed by rotary evaporation. The distillation residue is triturated with 250mL of diethyl ether and the resultant precipitates are filtered and dried at reduced pressure to give 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-ethanediamine amide, **48**.

Preparation of disulfide adduct, **50**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 3.50g (10mmol) of 3,5-(2-ditetrahydrofuranyloxy)-phenylacetic acid, N-ethanediamine amide, **48**, 100mL of dry 1,2-dimethoxyethane, 1.40mL (10mmol) of triethylamine, and 3.12g (10mmol) of N-succinimidyl-3-(2-pyridyldithio)-propionate, **49** (Pierce; Rockford, IL). The reaction mixture is stirred for 14 hour at room temperature, filtered and the solvents are removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to afford disulfide adduct, **50**.

Preparation of dihydroxyphenyl-disulfide adduct, **51**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 100mL of N,N-dimethylformamide (DMF), 5.47 g (10mmol) of disulfide adduct, **50**, and 11.97g of Fmoc-L-Cys-L-Glu(OtBu)-L-Glu(OtBu)-L-Glu(OtBu)-L-Leu-2-(5-fluorouracil-1-yl)-L,D-Gly-OH, **42**. The reaction mixture was allowed to stir at room temperature for 20h and then the solvents are removed at reduced pressure. The distillation residue is dissolved in a 1:1 mixture of water: DMF and then trifluoroacetic acid (TFA) is added to attain a pH of 5. The reaction mixture is stirred at room temperature for 2h and then the pH is adjusted to pH 7 using concentrated aqueous sodium carbonate solution. 100mL of dichloromethane is added, the organic layer is separated from the aqueous layer and the aqueous layer is extracted with a further 2x100mL of dichloromethane. The organic layers are combined, dried over anhydrous sodium sulfate for 4h, filtered, and the solvents are removed by rotary evaporation. The distillation residue is crystallized using an appropriate solvent(s) to yield dihydroxyphenyl disulfide adduct, **51**.

Preparation of invented drug polymer construct, **53**

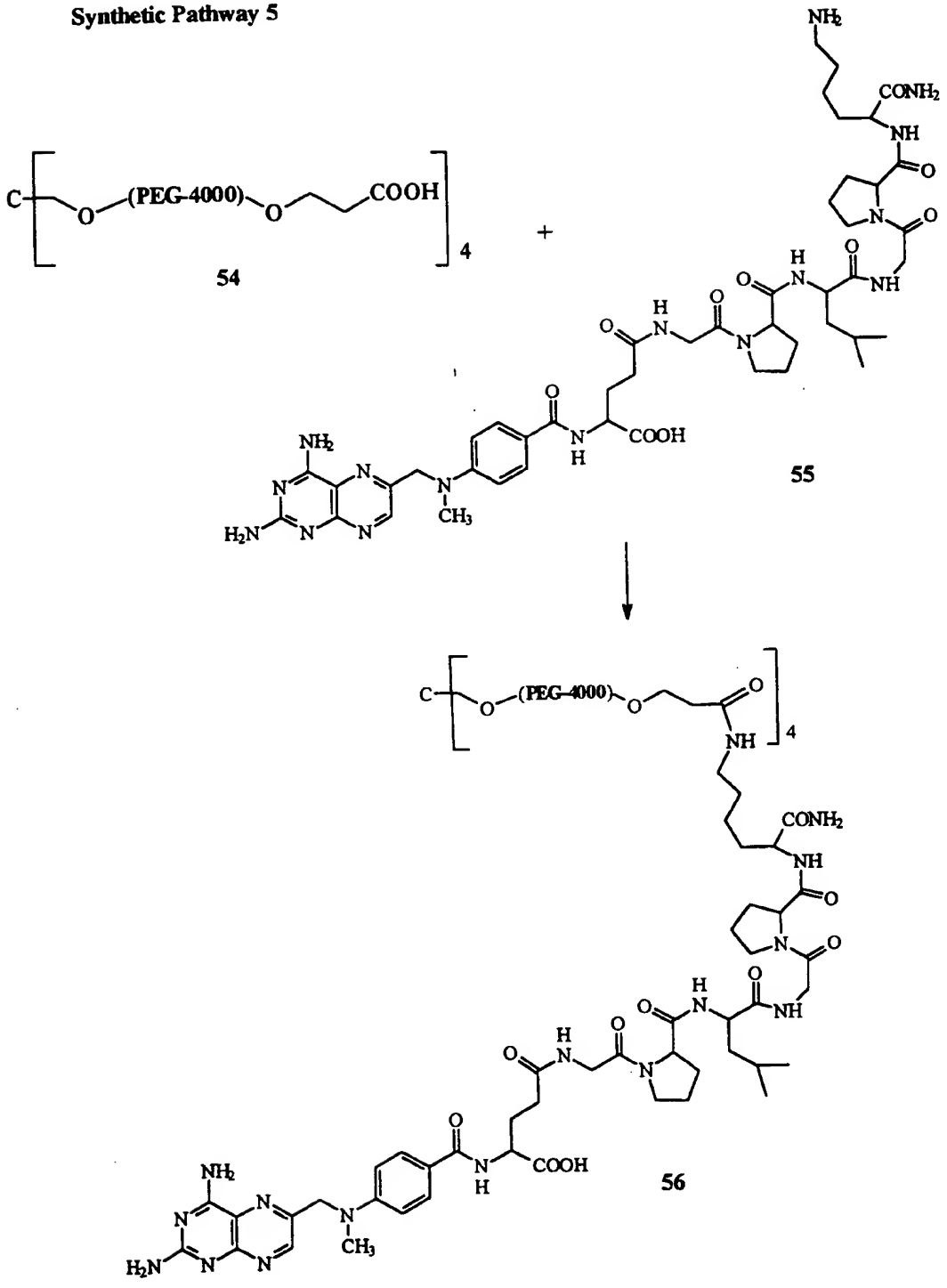
A 500mL round bottom flask equipped with a thermometer, addition funnel, condenser, magnetic stir bar, and dry N_2 inlet-outlet is charged with 100mL of acetonitrile, 0.28g (2mmol) of finely powdered potassium carbonate, 0.25g of 18-crown-6 (Aldrich), 10mL of water, and 1.49g (1mmol) of dihydroxyphenyl-disulfide adduct, **51**. The reaction mixture is heated to reflux and 2.0g of **DTS-PEG1000**, **52**, (see synthesis of ditosylate, **8**, above) is then added. The reaction mixture is heated to reflux, allowed to stir for 18h at room temperature, and then 0.1N HCl is slowly added to acidify the reaction mixture. The solvents are removed by rotary evaporation and 5mL of piperidine dissolved in 25mL of N,N-dimethylformamide (DMF) is added. The reaction mixture is stirred at room temperature for 1h and the solvents are removed by rotary evaporation. The distillation residue is dissolved in 5mL of trifluoroacetic acid (TFA) and 30mL of water and then the solvents are removed by rotary evaporation. The distillation residue is dissolved in 25mL of water and dialyzed against distilled water (Spectrapor membrane with molecular weight cutoff of 12,000-14,000). The resultant solution is lyophilized to give invented drug polymer conjugate, **53**. The average molecular weight of **53** is determined by GPC analysis and the degree of substitution of **51** into the product

construct is calculated using UV and NMR spectroscopy.

4.5 Assembly of Invented Drug Linked Polymer Conjugate, 56

The present example describes the preparation (see Synthetic Pathway 5) of a branched polymeric drug conjugate of the invention in which the pharmaceutical agent, **D**, is derived from methotrexate; the enzymatically cleavable region of the linker, (L_1-L_n), is hexapeptide, Gly-Pro-Leu-Gly-Pro-Lys-NH_2, the common multifunctional chemical moiety, **Q**, is pentaerythritol, and the water soluble polymer segment, **P**, is poly(ethylene glycol) with an average M.W. of about 4000.

Synthetic Pathway 5



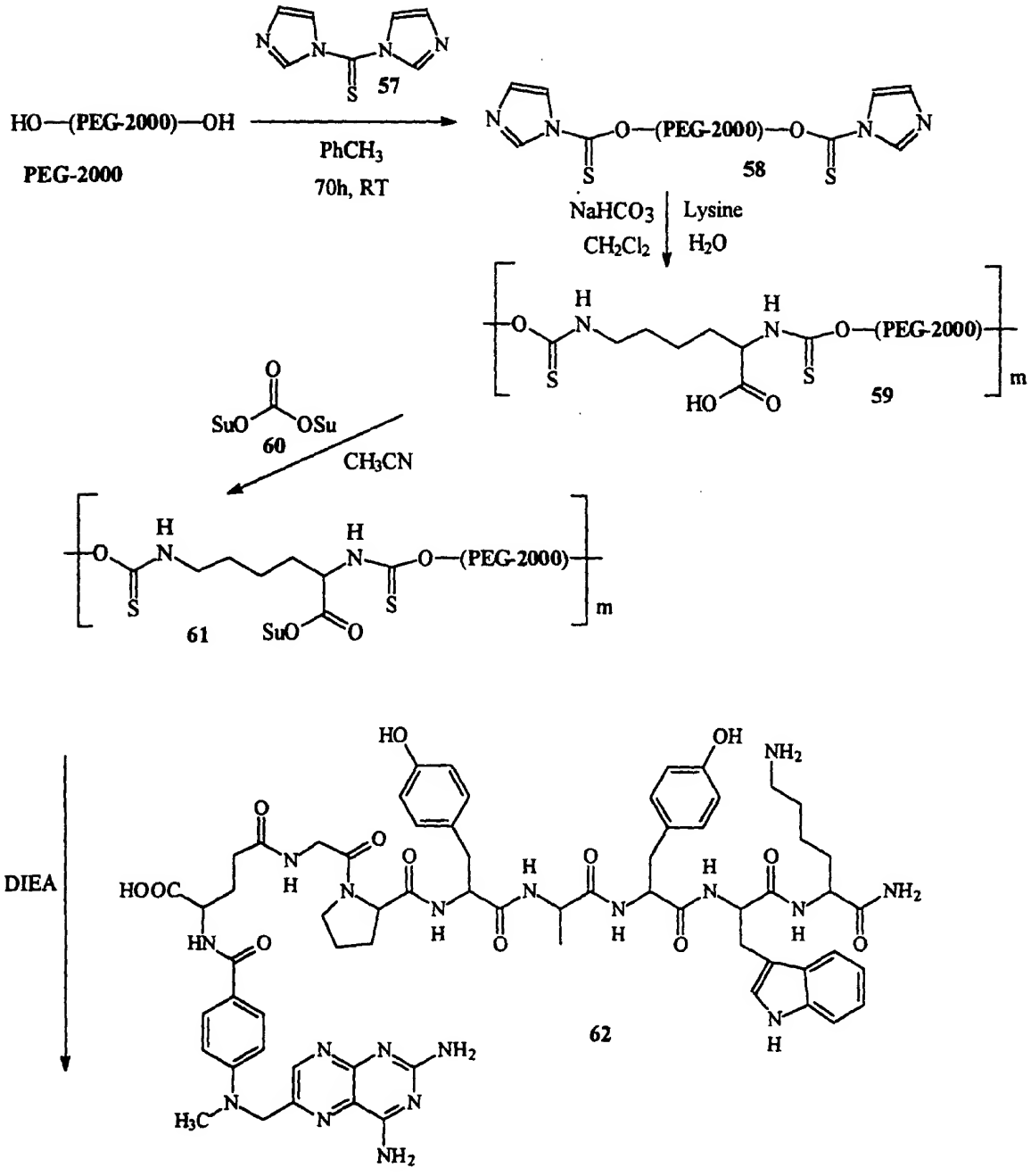
Preparation of invented drug polymer construct, **56**

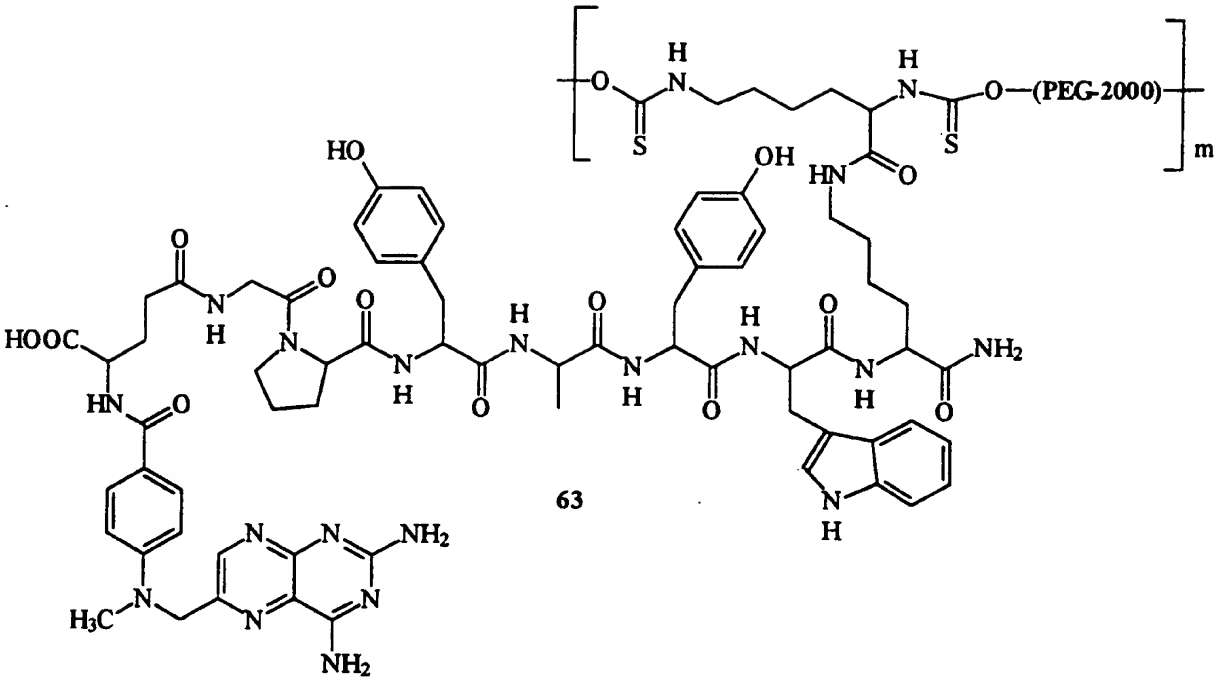
A clean dry 250mL round bottom flask equipped with a magnetic stir bar, thermometer, and Drierite filled drying tube is charged with 10mmol of 4 arm branched PEG-propionic acid system, **54** (Shearwater Polymers, Huntsville, AL), 100mL of dry N,N-dimethylformamide, 7.0 mL (50mmol) of triethylamine (Aldrich), and 40mmol of **55** (Anaspec, San Jose, CA). The reaction mixture is stirred for ten hours at room temperature and then poured into a 400mL of ice-cold water. A sufficient amount of 0.1N HCl is slowly added to bring the pH to 4 and the solvents are removed under reduced pressure. The resultant precipitates are crystallized from an appropriate solvent to give invented drug polymer construct, **56**. The average molecular weight of **56** is determined by GPC analysis and the degree of substitution of **55** onto the product construct is calculated using UV and NMR spectroscopy.

4.6 Assembly of Drug Linked Polymer Conjugate, 63

The present example (*see* Synthetic Pathway 6 below) describes the preparation of a regular repeating linear polymeric drug conjugate of the invention in which the biologically active agent, **D**, is methotrexate, the enzymatically cleaved region of the linker, ($\mathbf{L_1}$-$\mathbf{L_n}$), is the heptapeptide, Gly-Pro-Tyr-Ala-Tyr-Trp-Lys, the multifunctional chemical moiety, **M**, is lysine, and the water soluble polymer segment, **P**, is poly(ethylene glycol) with an average MW of about 2000 (**PEG-2000**).

Synthetic Pathway 6





63

Preparation of the bis(imidazolyl) thiocarbamate of poly(ethylene glycol), **58**

A clean dry 250mL round bottom flask equipped with a magnetic stir bar, heating mantle, Dean-Stark water removal trap, condenser, and Drierite filled drying tube ischarged with 120mL of toluene and 10.0g (5mmol) of **PEG-2000** (Aldrich, Milwaukee, WI). The resultant solution is refluxed for 2 hours, during which time approximately 100μL of water is collected in the Dean-Stark trap, and the heating mantle is then removed. The reaction mixture was allowed to cool to ambient temperature and 6.0g (33.7mmol) of 1,1'-thiocarbonyldiimidazole, tech., 90% (Aldrich) is added in one portion. The reaction mixture is stirred at RT for 70h and approximately ¾ of the solvent is then removed by rotary evaporation to give an orange oil. The oil is slowly added to 300mL of rapidly stirring ethyl ether to give a two phase mixture. The ethereal (upper) layer is decanted and the viscous oil in the lower layer is slowly added to 300mL of fresh ethyl ether. The ethereal layer is again decanted and the oily lower layer is dissolved in 70mL of ethyl acetate. The ethyl acetate solution is slowly dropped into 300mL of rapidly stirring cold ethyl ether to give off-white precipitates. The precipitated solids are filtered, air dried for 5 minutes, and allowed to stand for 4h under vacuum, to give 6.9g (3.1mmol, 62% yield) of the bis(imidazolyl) thiocarbamate of poly(ethylene glycol), **58**, as an off-white solid. ^{1}H NMR shows both aliphatic ethylene glycol protons and protons associated with the aromatic imidazole substituents.

Preparation of linear copolymer backbone, **59**

A clean dry 2mL reaction vial equipped with a magnetic spin vane is charged with 0.5mL of water, 0.8mL of methylene chloride, 29mg (345μmol) of sodium bicarbonate, 10mg (69μmol) of lysine (Aldrich), and 138mg (69μmol) of the bis(imidazolyl) thiocarbamate of poly(ethylene glycol), **58**. The reaction mixture is stirred at room temperature for 20h, the methylene chloride is removed by rotary evaporation and the remaining aqueous residue is washed with 3x1mL of methylene chloride. The organic layers are combined, dried over anhydrous magnesium sulfate, filtered, and the solvents are removed under reduced pressure. The distillation residue is dissolved in 3mL of deionized water and dialyzed (Spectrapor membrane with molecular weight cutoff of 3,500) against distilled water. The aqueous solution remaining in the dialysis bag is lyophilized to afford 64mg of linear copolymer backbone, **59**, as a white solid. GPC (Polymer Lab PL-GEL 10^3 and 10^5 column, 0.1% LiBr

in DMF eluent) shows the product copolymer to have a M_w of 19,022 with a polydispersity of 1.42.

Preparation of activated copolymer backbone, **61**

A 50mL round bottom flask equipped with a thermometer, magnetic stir bar and Drierite filled drying tube is charged with 20mL of acetonitrile, 4.74g (2.0mmol) of linear copolymer backbone, **59**, 1.0mL of pyridine, and 1.54g (6.0mmol) of N, N'-disuccinimidyl carbonate, tech. (Aldrich). The resultant solution is magnetically stirred at room temperature for 17h and then added dropwise to 300mL of rapidly stirring ethyl ether. The ethereal mixture is stirred for 0.5h, filtered, and the collected precipitates are washed with 2x300mL of fresh ether. The isolated solids are dried under vacuum to give activated copolymer backbone, **61**

Preparation of invented drug linked polymer construct, **63**

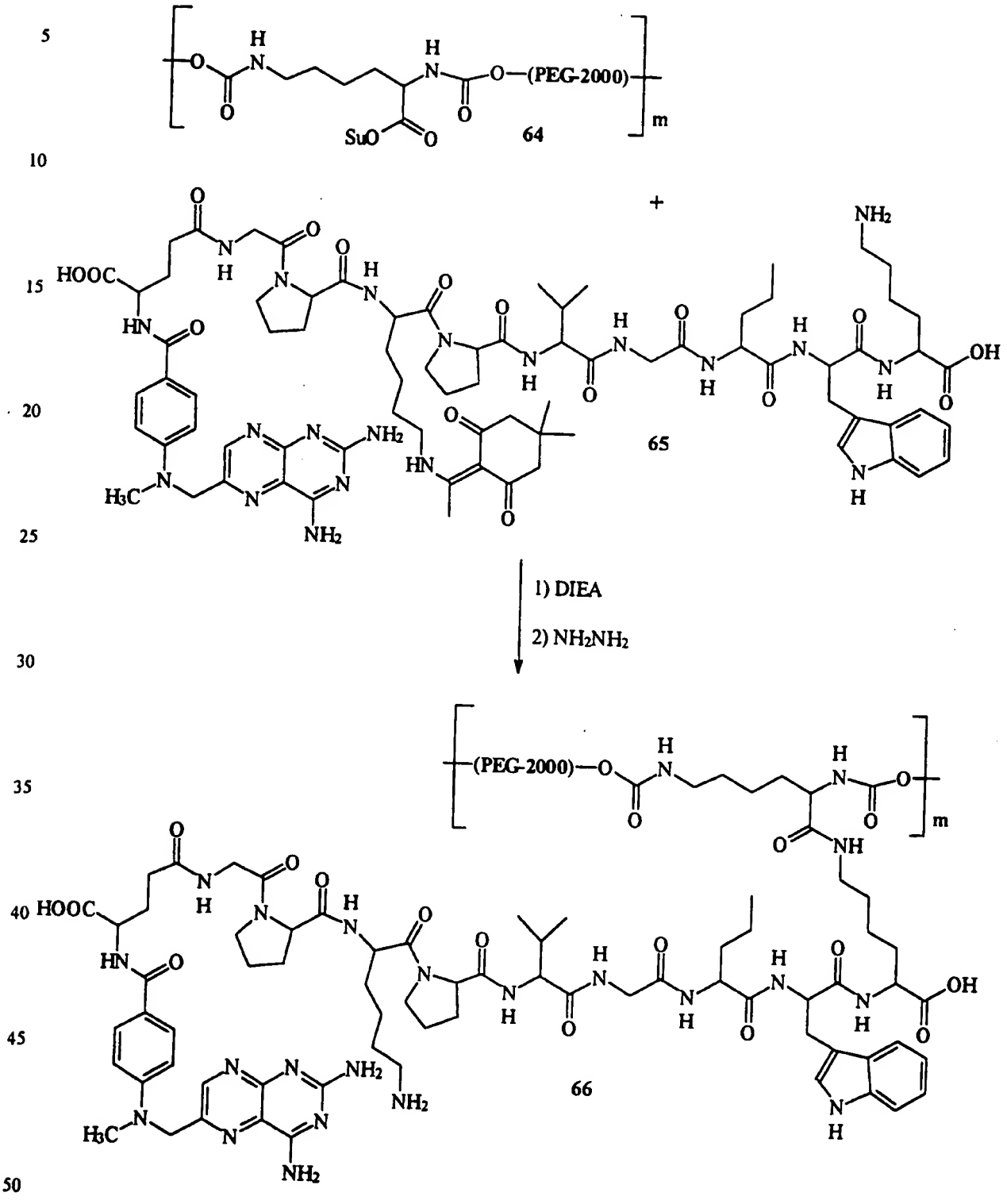
A 5mL reaction vial equipped with a magnetic spin vane is charged with 500μL of N,N-dimethylformamide, 117mg (50μmol) of activated copolymer backbone, **61**, 87μL (500μmol) of N,N-diisopropylethylamine (ChemImpex, Wood Dale, IL), and 66mg (50μmol) of peptide conjugate, **62**, (AnaSpec, Inc., San Jose, CA). The reaction mixture is stirred for 24h at room temperatutre and then added dropwise to 7mL of ethyl ether. The ethereal supernatant is decanted from the yellow precipitates and the remaining solids are washed with 7mL of fresh ethyl ether The collected solids are dissolved in 7.5mL of deionized water, the pH is adjusted to 2.5 by adding concentrated hydrochloric acid, and the resultant solution is dialyzed (Spectrapor membrane with molecular weight cutoff of 3,500) against distilled water. The aqueous solution is lyophilized to give the drug linked polymer constuct, **63**. The average molecular weight of **63** is determined by GPC analysis, the presence of the thiocarbamate linkages is confirmed by IR spectroscopy, and the degree of substitution of **62** onto the product construct is calculated using UV and NMR spectroscopy.

4.7 Assembly f Drug Linked Polymer C njugate, 67

The present example describes the preparation (*see* Synthetic Pathway 7) f a regular

repeating linear polymeric drug conjugate in which the pharmaceutical agent, **D**, is methotrexate, the enzymatically cleaved region of the linker, (L_1-L_n), is the peptide, Gly-Pro-Lys-Pro-Val-Gly-Nva-Trp-Lys, the multifunctional chemical moiety, **M**, is lysine, and the water soluble polymer segment, **P**, is poly(ethylene glycol) with an average MW of about 2000 (**PEG-2000**).

Synthetic Pathway 7



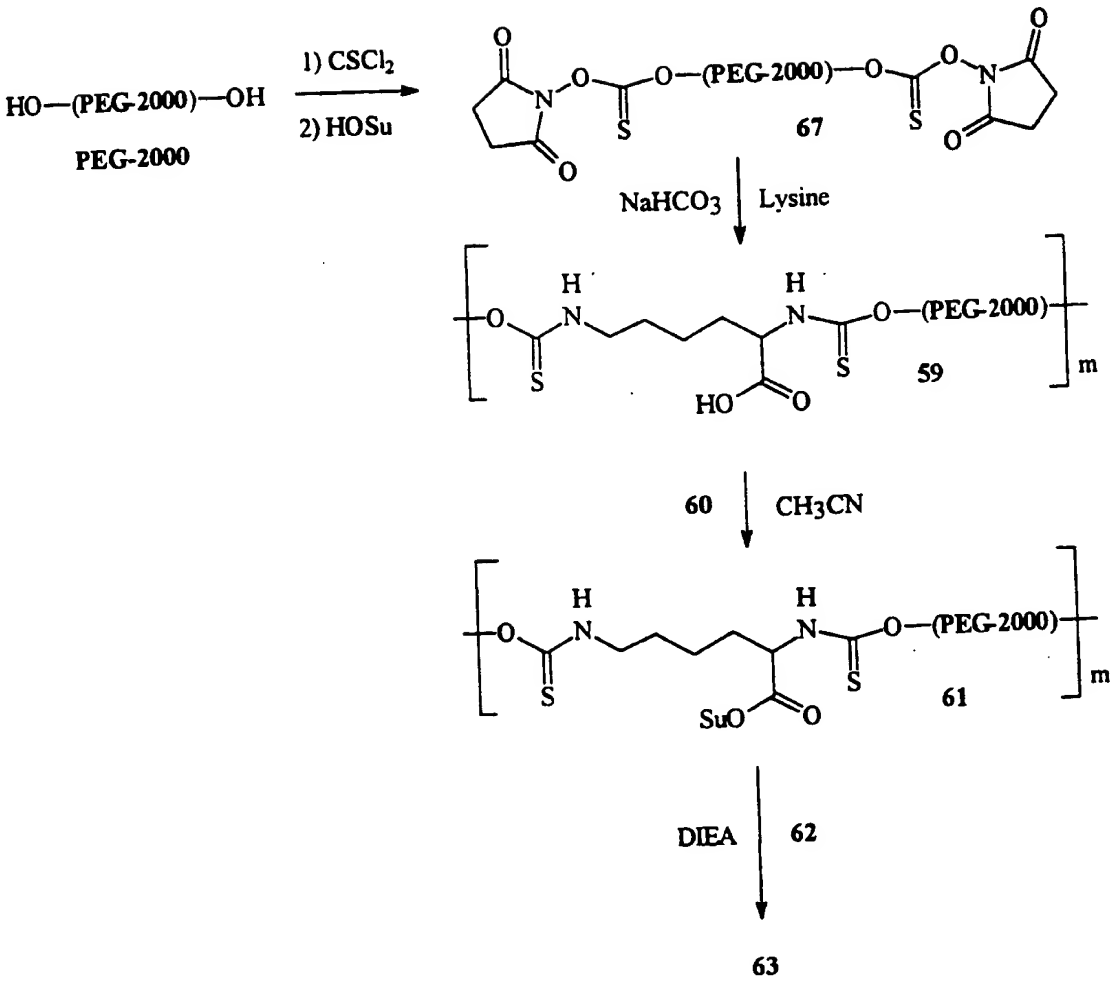
Preparation of invented drug linked polymer construct, **66**

A 5mL reaction vial equipped with a magnetic spin vane is charged with 1mL of N,N-dimethylformamide, 74.8mg (32μmol) of activated copolymer backbone, **64** (prepared by the method of Kohn, *et al.*, Macromolecules, 1992, *25*, 4476), 55μL (320μmol) of N,N-diisopropylethylamine (ChemImpex, Wood Dale, IL), and 50mg (32μmol) of peptide conjugate, **65** (AnaSpec, Inc., San Jose, CA). The reaction mixture is stirred for 60h at room temperature and then 5mL of water is added. The aqueous solution is stirred for 24h at room temperature, 200μL of hydrazine (Aldrich) is added, and the resultant aqueous solution is dialyzed (Spectrapor membrane with molecular weight cutoff of 3,500) against distilled water. The homogeneous solution in the dialysis membrane is lyophilized to give the drug linked polymer constuct, **66**. The average molecular weight of **66** is determined by GPC analysis, the presence of the carbamate linkages is confirmed by IR spectroscopy, and the degree of substitution of **65** onto the product is calculated using UV and NMR spectroscopy.

4.8 Alternate Assembly of Drug Linked Polymer Conjugate, 63

The present example describes the preparation (*see* Synthetic Pathway 8 below) of a regular repeating linear polymeric drug conjugate of the invention in which the biologically active agent, **D**, is methotrexate, the enzymatically cleaved region of the linker, (L_1-L_n), is the heptapeptide, Gly-Pro-Tyr-Ala-Tyr-Trp-Lys, the multifunctional chemical moiety, **M**, is lysine, and the water soluble polymer segment, **P**, is poly(ethylene glycol) with an average MW of about 2000 (**PEG-2000**).

Synthetic Pathway 8



Preparation of the bis-succinimidylthiocarbonate of poly(ethylene glycol), **67**

A clean dry 250mL round bottom flask equipped with a condenser, magnetic stir bar, addition funnel, drying tube, heating mantle, Dean-Stark water removal trap, and HCl trap is charged with 10.0g (5mmol) of **PEG-2000** (Aldrich) and 120mL of toluene. The polymer solution is azeotropically dried for 2h under reflux, cooled to room temperature, and then 2.2mL (29mmol) of thiophosgene (Aldrich) is added in one portion. The reaction mixture is allowed to stir for 18h at room temperature and then the solvents are removed at reduced pressure. An additional 100mL of toluene is added to the distillation residue and the solvents are again evaporated under reduced pressure. The distillation residue is dissolved in a 3:1 mixture of toluene:methylene chloride, 1.7g (14.8mmol) of N-hydroxy succinimide is added, and the reaction mixture is stirred at room temperature for 1h. The reaction mixture is cooled in an ice-water bath and 1.5g (14.9mmol) of triethylamine (Aldrich) is added. The cooling bath is removed, the reaction mixture is allowed to stir at room temperature for 5h, the ice-water bath is again applied, and the reaction mixture is filtered. Approximately half of the solvents are removed from the filtrate by rotary evaporation and 60mL of ethyl ether is added with vigorous stirring. The resultant precipitates are filtered and crystallized using an appropriate solvent to give the bis-succinimidylthiocarbonate of poly(ethylene glycol), **67**. NMR and IR spectroscopy is used to identify and quantify the degree of thiocarbonate formation.

Preparation of linear repeating copolymer, **59**

A clean 500mL three-necked round-bottom flask equipped with an overhead stirrer is charged with 1.1g (6.8mmol) of L-lysine (Aldrich), 2.5g (31.5mmol) of sodium bicarbonate and 150mL of water. A solution consisting of 6.8mg of **67** dissolved in 300mL of methylene chloride is added and the reaction mixture is stirred vigorously for 2h at room temperature. The pH is lowered to 2 by the careful addition of concentrated hydrochloric acid and the organic layer is separated from the aqueous layer. The organic layer is washed with 2x100mL of saturated aqueous sodium chloride solution, dried over anhydrous magnesium sulfate, filtered, and the solvents are removed by rotary evaporation. The crude polymer is dissolved in 50mL of water and dialyzed against distilled water using a SPECTRAPOR membrane with

a molecular weight cut-off of 12,000-14,000 daltons. The aqueous solution remaining in the dialysis membrane is lyophilized to give linear repeating copolymer, **59**, as a white solid. GPC is used to determine the average molecular weight of the product, IR spectroscopy confirms the presence of thiocarbamate linkages, and ^{1}H NMR spectroscopy is used to confirm the presence of lysine protons.

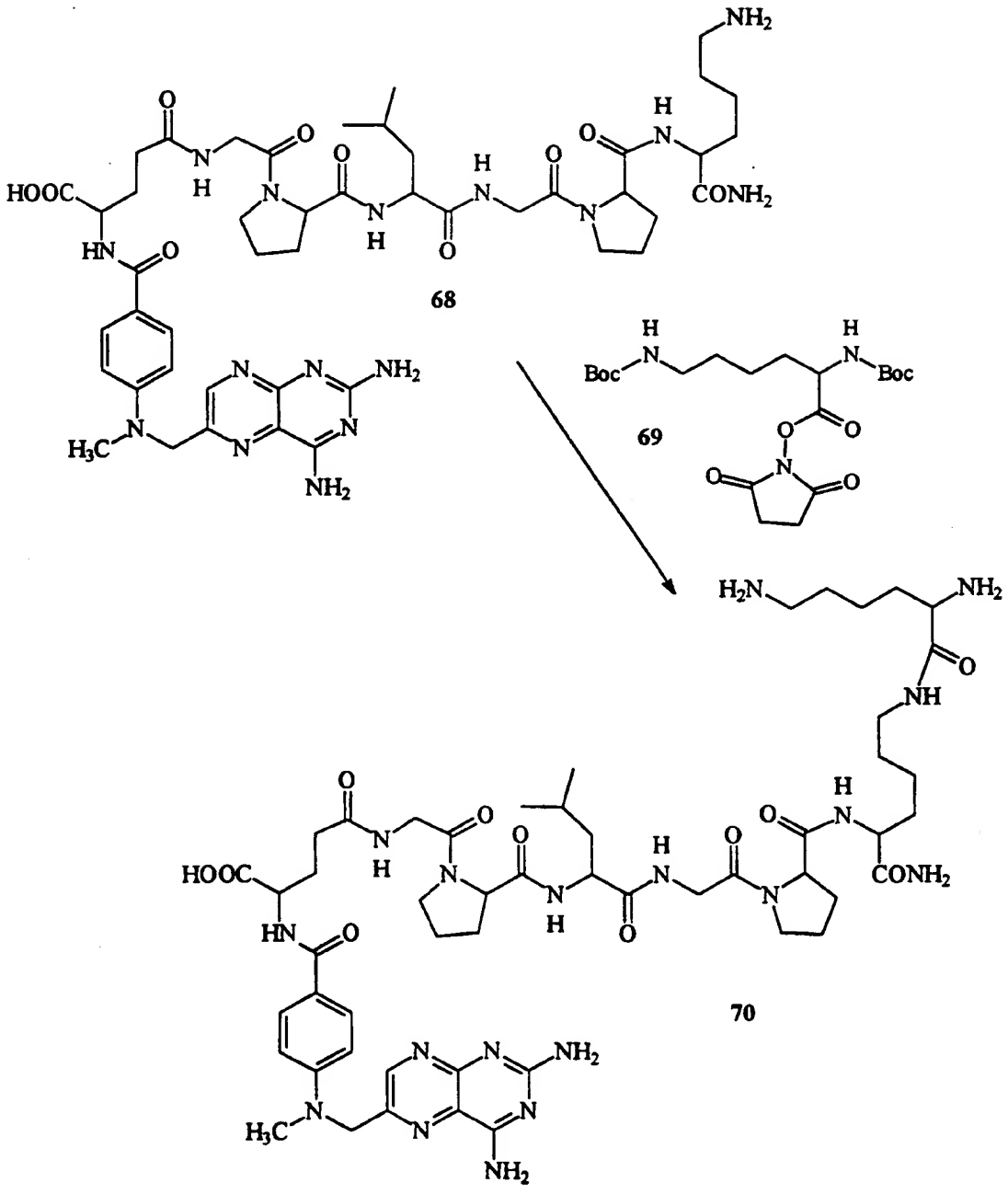
Preparation of invented drug linked polymer conjugate, **63**

The two step procedure used for the preparation of invented construct **63** using linear repeating polymer **59** is the same at that described in Section 4.6.

4.9 Assembly of Drug Linked Polymer Conjugate, 71

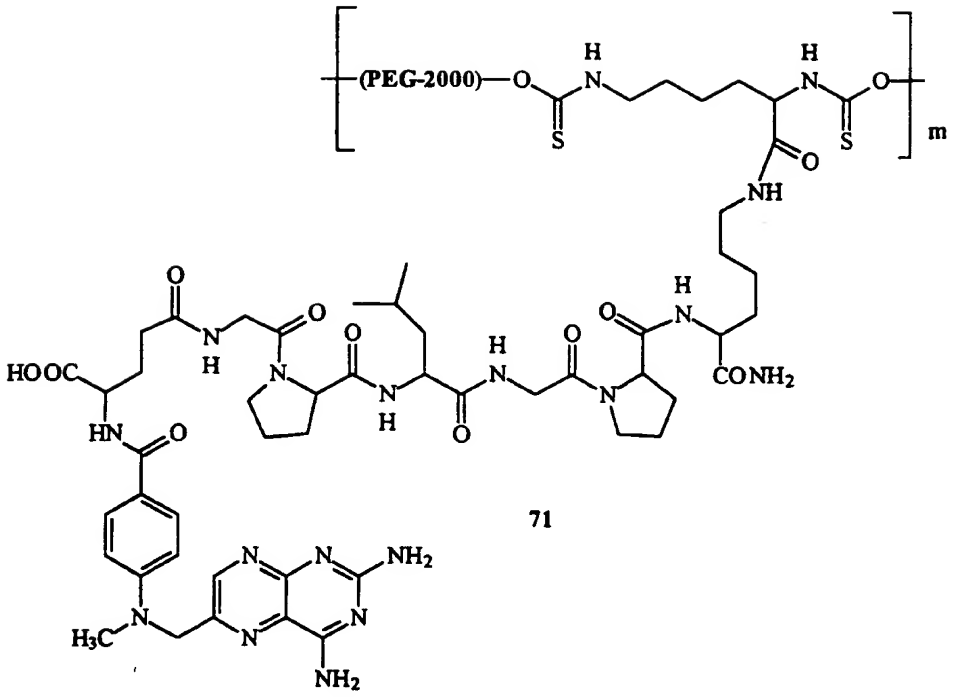
The present example describes the preparation (*see* Synthetic Pathway 9) of a regular repeating linear polymeric drug conjugate of the invention in which the pharmaceutical agent, **D**, is methotrexate, the enzymatically cleaved region of the linker, (L_1-L_n), is hexapeptide, Gly-Pro-Leu-Gly-Pro-Lys, the multifunctional chemical moiety, **M**, is lysine, and the water soluble polymer segment, **P**, is poly(ethylene glycol) (**PEG-2000**) with an average MW of about 2000.

Synthetic Pathway 9



70

58



71

Preparation of methotrexate-peptide conjugate, **70**

A clean dry 50mL reaction vial equipped with a magnetic stir bar and drying tube is charged with 25mL of N,N-dimethylformamide, 1.00g (1mmol) of methotrexate-peptide conjugate, **68**, (AnaSpec, Inc.), 1.74mL (10mmol) of N,N-diisopropylethylamine (Aldrich), and 0.44g (1mmol) of Nα,Nε-di-t-Boc-L-lysine, N-hydroxysuccinimide ester (Sigma, St. Louis, MO). The reaction mixture is allowed to stir at room temperature for 18h and then the solvents are removed by rotary evaporation. The distillation residue is dissolved in water and the desired product is isolated by reverse phase C-18 chromatography using acetonitrile:water (0.1%TFA) elution. The product containing eluent fractions are combined and lyophilized to give methotrexate-peptide conjugate, **70**, as a yellow solid. The identity of the product is determined by ^{1}H NMR and mass spectrometry.

Preparation of the invented drug linked polymer construct, **71**

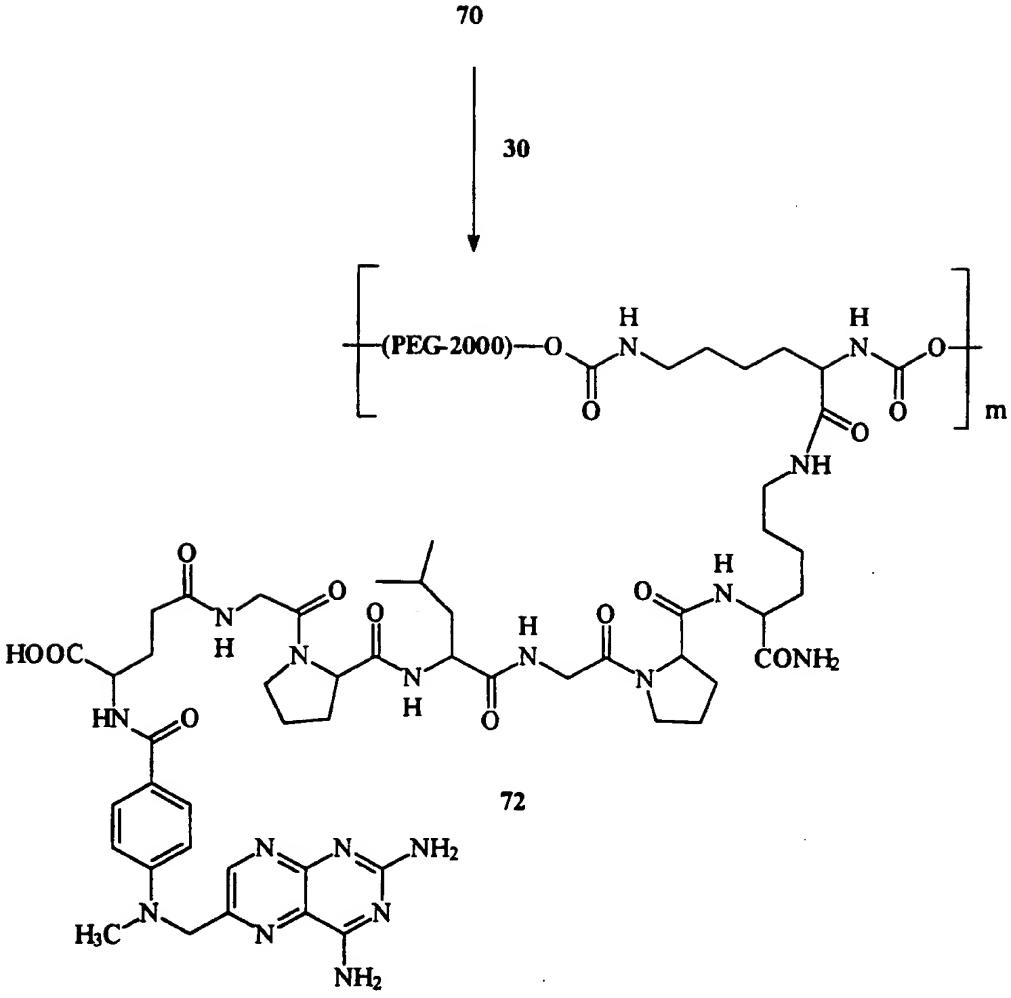
A clean dry 50mL reaction vial equipped with a magnetic stir bar and drying tube is charged with 25mL of N,N-dimethylformamide, 1.13g (1mmol) of methotrexate-peptide conjugate, **70**, 1.74mL (10mmol) of N,N-diisopropylethylamine (Aldrich), and 2.22g (1mmol) of the bis(imidazolyl) thiocarbamate of poly(ethylene glycol), **58**. The reaction mixture is allowed to stir at room temperature for 18h and then the solvents are removed by rotary evaporation. The distillation residue is dissolved in water and the resultant solution is dialyzed (Spectrapor membrane with molecular weight cutoff of 10,000) against distilled water to give the invented drug linked polymer construct **71** as a yellow solid. The average molecular weight of the construct is determined by GPC, the presence of the thiocarbamate linkages is confirmed by IR spectroscopy, and the degree of substitution of **70** into the product construct is calculated using UV and NMR spectroscopy.

4.10 Assembly of Drug Linked Polymer Conjugate, 72

The present example describes the preparation (*see* Synthetic Pathway 10) of a regular repeating linear polymeric drug conjugate of the invention in which the pharmaceutical agent, **D**, is methotrexate, the enzymatically cleaved region of the linker, (L_1-L_n), is hexapeptide, Gly-Pro-Leu-Gly-Pro-Lys, the multifunctional chemical moiety, **M**, is lysine, and the water soluble polymer segment, **P**, is poly(ethylene glycol) (**PEG-2000**) with an average MW of about 2000.

Synthetic Pathway 10

70



72

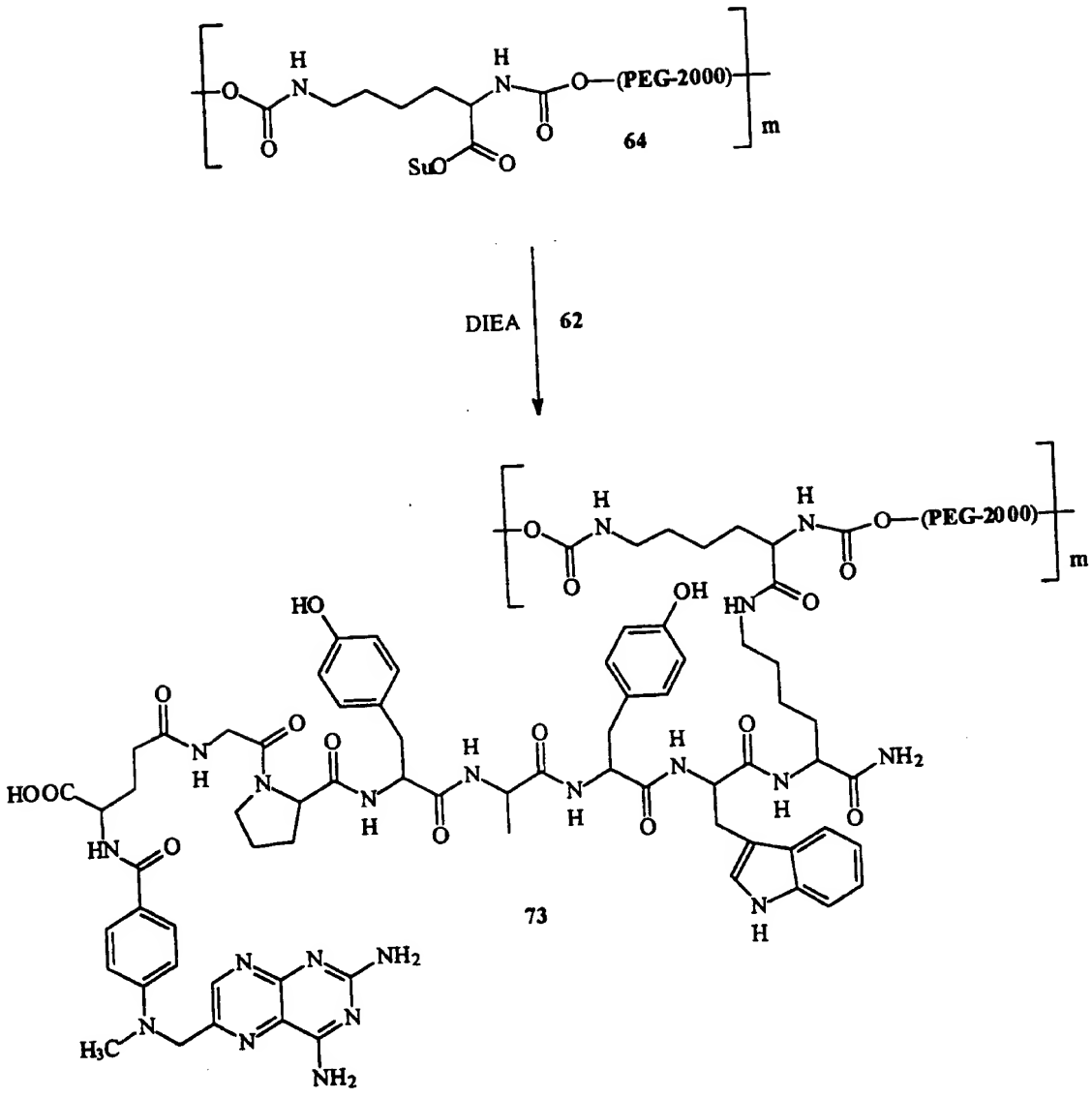
Preparation of the invented drug linked polymer construct, **72**

A clean dry 50mL reaction vial equipped with a magnetic stir bar and drying tube is charged with 25mL of N,N-dimethylformamide, 1.13g (1mmol) of methotrexate-peptide conjugate, **70**, 1.74mL (10mmol) of N,N-diisopropylethylamine (Aldrich), and 2.28g (1mmol) of the bis-succinimidylcarbonate of poly(ethylene glycol) with an average molecular weight of 2000, **30**. The reaction mixture is allowed to stir at room temperature for 18h and then the solvents are removed by rotary evaporation. The distillation residue is dissolved in water, the pH is adjusted to 2.5 using conc. HCl, and the resultant solution is dialyzed (Spectrapor membrane with molecular weight cutoff of 10,000) against distilled water to give the invented drug linked polymer construct **72** as a yellow solid. The average molecular weight of the construct is determined by GPC, the presence of the thiocarbamate linkages is confirmed by IR spectroscopy, and the degree of substitution of **70** into the product is calculated using UV and NMR spectroscopy.

4.11 Assembly of Drug Linked Polymer Conjugate, 66

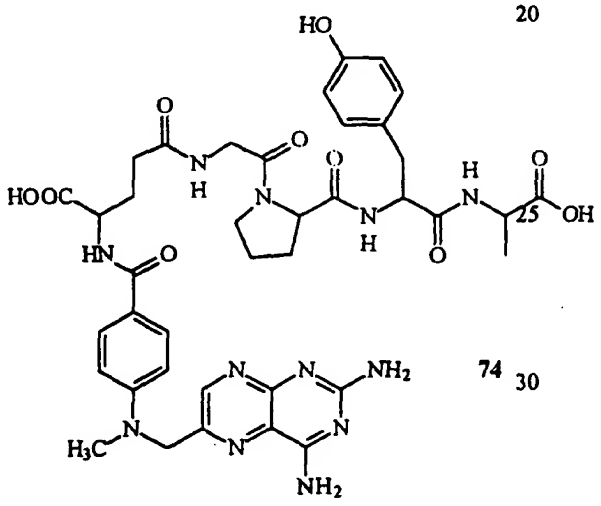
The present example describes the preparation (*see* Synthetic Pathway 11) of a regular repeating linear polymeric drug conjugate of the invention in which the pharmaceutical agent, **D**, is methotrexate, the enzymatically cleaved region of the linker, (L_1-L_n), is heptapeptide, Gly-Pro-Tyr-Ala-Tyr-Trp-Lys, the multifunctional chemical moiety, **M**, is lysine, and the water soluble polymer segment, **P**, is poly(ethylene glycol) (**PEG-2000**) with an average MW of about 2000.

Synthetic Pathway 11



Preparation of invented drug linked polymer construct, 73

A 5mL reaction vial equipped with a magnetic spin vane is charged with 0.5mL of N,N-dimethylformamide, 117mg (50μmol) of activated copolymer backbone, 64, (prepared by the method of Kohn, *et al.*, Macromolecules, 1992, *25*, 4476), 87μL (10equiv.) of N,N-diisopropylethylamine (ChemImpex), and 50mg (50μmol) of peptide conjugate, 62 (AnaSpec). The reaction mixture is stirred for 24h at room temperature and added dropwise to 7mL of ethyl ether to give yellow precipitates. The liquid is decanted from the precipitates and the solids are washed with 7mL of fresh ethyl ether. The supernatant is again decanted, the solid residue is dried at reduced pressure, and 7.5mL of deionized water is added. The resultant aqueous solution is dialyzed (Spectrapor membrane with molecular weight cutoff at 3,500 daltons) against deionized water and lyophilized to give 95mg (28.5mmol, 57%yield) of invented drug linked construct, 73, as a yellow solid. GPC (Polymer Lab PL-GEL 10,000 and 100,000 column with DMF, 0.1% LiBr as eluent) shows the average molecular weight of the polymer-drug construct to be 73,072 with a polydispersity of 4.17. The extent of incorporation of 62 onto the PEG-lysine backbone is determined to be 39% by UV spectroscopy. Treatment of 73 with MMP3 enzyme (Chemicon International, Temecula, CA) results in the formation of methotrexate-peptide fragment 74. The rate of cleavage of 74 from 73 is determined by RP-18 HPLC to be 33% over 2h at 37 °C.



74

Cytotoxicity studies using U973 cells showed polymer construct **73** allowed cell growth equivalent to that of the control (designated as 100%). Samples of methotrexate-peptide fragment, **74**, and methotrexate alone allowed 10% and 6% cell growth respectively.

All publications, patents, and patent documents referred to herein are hereby incorporated in their respective entireties by reference.

The invention has been described with reference to the foregoing specific and preferred embodiments and methods. However, it should be understood that many variations may be made while remaining within the spirit and scope of the invention. Therefore, the foregoing examples are not limiting, and the scope of the invention is intended to be limited only by the following claims.

CLAIMS

1. A polymeric drug conjugate comprising one or more biologically active agents conjugated via an enzymatically cleavable linker to:
   (i) a regular repeating linear unit comprising a water soluble polymer segment and a multifunctional chemical moiety, or
   (ii) a branched polymer comprising two or more water soluble polymer segments each bound to a common multifunctional chemical moiety.

2. The conjugate of claim 1, in which said one or more biologically active agents are conjugated via said linker to said multifunctional chemical moiety of said regular repeating linear unit.

3. The conjugate of claim 1, in which said one or more biologically active agents are conjugated via said linker to at least one of said two or more water soluble polymer segments.

4. The conjugate of claim 1, in which said linker is cleaved by an intracellular enzyme.

5. The conjugate of claim 1, in which said linker is cleaved by an extracellular enzyme.

6. The conjugate of claim 1, in which said linker is cleaved by a membrane-bound enzyme.

7. The conjugate of claim 1, in which said linker is cleaved by an enzyme that is available at a target site.

8. The conjugate of claim 7, in which said enzyme is up-regulated at said target site.

9. The conjugate of claim 7, in which said target site is diseased tissue or biological fluid.

10. The conjugate of claim 9, in which said diseased tissue is present in skin, bone, cartilage, muscle, connective tissue, neural tissue, reproductive organs, endocrine tissue, lymphatic tissue, vasculature, or visceral organs.

11. The conjugate of claim 9, in which said biological fluid is blood, pleural fluid, peritoneal fluid, joint fluid, pancreatic fluid, bile, or cerebral-spinal fluid.

12. The conjugate of claim 1, in which the linker is cleaved by an enzyme resulting from a microbial infection, a skin surface enzyme, or an enzyme secreted by a cell.

13. The conjugate of claim 1, in which said linker is cleaved by an enzyme secreted by a cancer cell.

14. The conjugate of claim 1, in which said linker is cleaved by an enzyme located on the surface of a cancer cell.

15. The conjugate of claim 1, in which said linker is cleaved by an secreted by a cell associated with a chronic inflammatory disease.

16. The conjugate of claim 1, in which said linker is cleaved by an enzyme secreted by a cell associated with rheumatoid arthritis.

17. The conjugate of claim 1, in which said linker is cleaved by an enzyme secreted by a cell associated with osteoarthritis.

18. The conjugate of claim 1, in which said linker is further cleaved by hydrolysis, reduction reactions, oxidative reactions, pH shifts, photolysis, or combinations thereof.

19. The conjugate of claim 1, in which said linker is further cleaved by a non-specific enzyme reaction.

20. The conjugate of claim 2, in which said multifunctional chemical moiety is derived from a group selected from N-(2-hydroxyacetyl)serine, lysine, tris(2-aminoethyl)amine, N-(*p* –nitrophenylacetyl)-*p*-nitrophenylalanine acid hydrazide, 3,5-dihydroxyphenylacetic acid, 3,5-diaminobenzoic acid, and 6-amino-4-(2-aminoethyl)hexanoic acid.

21. The conjugate of claim 3, in which said common multifunctional chemical moiety comprises pentaerythritol, dendrimers, or branched lysine trees.

22. The conjugate of claim 1, in which said water soluble polymer segment comprises a polymer with a molecular weight of about 400 to about 25,000.

23. The conjugate of claim 1, in which said water soluble polymer segment comprises poly(ethylene glycol), a copolymer of poly(ethylene glycol), or combinations thereof.

24. The conjugate of claim 1, in which said water soluble polymer segment comprises poly(vinyl alcohol), poly(2-hydroxyethyl methacrylate), poly(acrylic acid), poly(methacrylic acid), poly(maleic acid), poly(lysine), and the like, or combinations thereof.

25. The conjugate of claim 1, in which said linker comprises an amino acid, a sugar, a nucleic acid, or other organic compounds, or combinations thereof.

26. The conjugate of claim 1, in which said linker comprises a peptide sequence.

27. The conjugate of claim 1, in which said linker comprises a peptide sequence which can be cleaved by a serine protease.

28. The conjugate of claim 27, in which said serine protease is selected from the group consisting of thrombin, chymotrypsin, trypsin, elastase, kallikrein, and substilisin.

29. The conjugate of claim 28, in which said thrombin-cleavable peptide sequence comprises -Gly-Arg-Gly-Asp-, -Gly-Gly-Arg-, -Gly-Arg-Gly-Asp-Asn-Pro-, -Gly-Arg-Gly-Asp-Ser-, -Gly-Arg-Gly-Asp-Ser-Pro-Lys-, -Gly-Pro-Arg-, -Val-Pro-Arg-, or -Phe-Val-Arg-.

30. The conjugate of claim 28, in which said elastase-cleavable peptide sequence comprises -Ala-Ala-Ala-, -Ala-Ala-Pro-Val-, -Ala-Ala-Pro-Leu-, -Ala-Ala-Pro-Phe-, -Ala-Ala-Pro-Ala-, or -Ala-Tyr-Leu-Val-.

31. The conjugate of claim 1, in which said linker comprises a peptide sequence which can be cleaved by a cysteine proteinase.

32 The conjugate of claim 31, in which said cysteine proteinase is selected from the group consisting of papain, actinidin, bromelain, lysosomal cathepsins, cytosolic calpain, and parasitic protease.

33. The conjugate of claim 32, in which said parasitic protease is derived from *Trypanosoma* or *Schistosoma*.

34. The conjugate of claim 1, in which said linker comprises a peptide sequence which can be cleaved by an aspartic proteinase.

35. The conjugate of claim 34, in which said aspartic proteinase is selected from the group consisting of pepsin, chymosin, lysosomal cathepsins D, a processing enzyme, a fungal protease, and a viral proteinase.

35. The conjugate of claim 35, in which said processing enzyme comprises renin.

37. The conjugate of claim 35, in which said fungal protease comprises penicillopepsin, rhizopuspepsin, or endothiapepsin.

38. The conjugate of claim 35, in which said viral protease comprises the protease from the AIDS virus.

39. The conjugate of claim 1, in which said linker comprises a peptide sequence that can be cleaved by a matrix metalloproteinase.

40. The conjugate of claim 39, in which said matrix metalloproteinase is selected from the group consisting of collagenase, stromelysin, and gelatinase.

41. The conjugate of claim 39, in which said matrix metalloproteinase comprises -Gly-Pro-Y-Gly-Pro-Z-, -Gly-Pro-Leu-Gly-Pro-Z-, -Gly-Pro-Ile-Gly-Pro-Z-, or -Ala-Pro-Gly-Leu-Z-, where Y and Z are amino acids.

42. The conjugate of claim 39, in which said matrix metalloproteinase comprises -Leu-Gly-, or Ile-Gly.

43. The conjugate of claim 40, in which said collagenase-cleavable peptide sequence comprises -Pro-Leu-Gly-Pro-D-Arg-Z-, -Pro-Leu-Gly-Leu-Leu-Gly-Z-, -Pro-Gln-Gly-Ile-Ala-Gly-Trp-, -Pro-Leu-Gly-Cys(Me)-His-, -Pro-Leu-Gly-Leu-Trp-Ala-, -Pro-Leu-Ala-Leu-Trp-Ala-Arg-, or -Pro-Leu-Ala-Tyr-Trp-Ala-Arg-, where Z is an amino acid.

44. The conjugate of claim 40, in which said stromelysin-cleavable peptide sequence comprises -Pro-Tyr-Ala-Tyr-Trp-Met-Arg-.

45. The conjugate of claim 40, in which said gelatinase-cleavable peptide sequence comprises -Pro-Leu-Gly-Met-Trp-Ser-Arg-.

46. The conjugate of claim 1, in which said linker comprises a peptide sequence that can be cleaved by an angiotensin converting enzyme.

47. The conjugate of claim 46, in which said angiotensin converting enzyme comprises -Asp-Lys-Pro-, -Gly-Asp-Lys-Pro-, or -Gly-Ser-Asp-Lys-Pro-.

48. The conjugate of claim 1, in which said linker comprises a peptide sequence that can be cleaved by a prostate specific antigen or a prostate specific membrane antigen.

49. The conjugate of claim 48, in which said linker includes $-(Glu)_n-$, and n is an integer from 1 to 10.

50. The conjugate of claim 1, in which said biologically active agent comprises an analgesic, an anesthetic, an antifungal, an antibiotic, an antiinflammatory, an anthelmintic, an antiarthritic, an antidote, an antiemetic, an antihistamine, an antihypertensive, an antimalarial, an antimicrobial, an antipsychotic, an antipyretic, an antiseptic, an antiarthritic, an antituberculotic, an antitussive, an antiviral, a cardioactive drug, a cathartic, a chemotherapeutic agent, a corticoid, an antidepressant, a depressant, a diagnostic aid, a diuretic, an enzyme, an expectorant, a hormone, a hypnotic, a mineral, a nutritional supplement, a parasympathomimetic, a potassium supplement, a radiation sensitizer, a sedative, a sulfonamide, a stimulant, a sympathomimetic, a tranquilizer, a urinary antiinfective, a vasoconstrictor, a vasodilator, a vitamin, an xanthine derivative, or the like and combinations thereof.

51. The conjugate of claim 50, in which said chemotherapeutic agent comprises a nitrogen mustard, an ethylenimine, a methylmelamine, a nitrosourea, an alkyl sulfonate, a triazene, a folic acid analog, a pyrimidine analog, a purine analog, a vinca alkaloid, an epipodophyllotoxin, an antibiotic, an enzyme, a biological response modifier, a platinum complex, a methylhydrazine derivative, an adrenocorticol suppressant, a somatostatin, a somatostatin analog, a hormone, a hormone antagonist, or combinations thereof.

52. The conjugate of claim 51, in which said chemotherapeutic agent comprises methotrexate, taxol, aminopterin, doxorubicin, bleomycin, camptothecin, etoposide, estramustine, prednimustine, melphalan, hydroxyurea, or 5-fluorouracil.

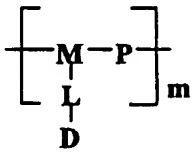
53. The conjugate of claim 1, in which said biologically active agent comprises a peptide based pharmaceutical agent.

54. The conjugate of claim 53, in which said peptide based pharmaceutical agent comprises a cytokine, a growth factor, a cell receptor antagonist, or a cell receptor agonist.

55. The conjugate of claim 1, in which said biologically active agent comprises an eptifibatide and other platelet binding proteins, a granulocyte colony stimulating factor, a human growth factor, a vascular endothelial growth factor, a bone morphogenic protein, an interferon, or an interleukin.

56. The conjugate of claim 1, in which said biologically active agent comprises DNA, RNA, a DNA fragment, an RNA fragment, or a plasmid.

57. The conjugate of claim 2, comprising the structure:



wherein P is said water soluble polymer segment, M is said multifunctional chemical moiety, L is said linker, D is said biologically active agent, and m is an integer.

58. The conjugate of claim 57, wherein m is an integer that is greater than or equal to 2.

59. The conjugate of claim 58, wherein m is an integer from about 2 to about 25.

60. The conjugate of claim 57, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, said multifunctional chemical moiety comprises N-(2-hydroxyacetyl)serine, said linker comprises (H-Leu-Gly-Pro-Ala-NH-CH_2-CH_2-NH_2), and said biologically active agent comprises doxorubicin-14-O-hemiglutarate.

61. The conjugate of claim 57, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 1,000, said multifunctional chemical moiety comprises tris(2-aminoethyl)amine, said linker comprises (H-Val-Pro-Arg-OH), and said biologically active agent comprises N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic (1→6)-disulfide.

62. The conjugate of claim 57, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, said multifunctional chemical moiety comprises N_{α}-(-*p*-aminophenylacetyl)-*p*-aminophenylalanyl hydrazide, said linker comprises (OCH-CO-Gly-Pro-Leu-Gly-Pro-OH), and said biologically active agent comprises a pharmaceutical analog of Leu-Gly-α-5-fluorouracil.

63. The conjugate of claim 57, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 1,000, said multifunctional chemical moiety comprises 3,5-dihydroxyphenylacetic acid, said linker comprises (H-Cys(S-CH_2-CH_2-CO-NH-CH_2-CH_2-NH_2)-Glu-Glu-Glu-OH), and said biologically active substance comprises a pharmaceutical analog of Leu-Gly-α-5-fluorouracil.

64. The conjugate of claim 57, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, said multifunctional chemical moiety comprises lysine, said linker comprises (H-Gly-Pro-Tyr-Ala-Tyr-Trp-Lys-NH_2), and said biologically active agent comprises methotrexate.

65. The conjugate of claim 57, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, said multifunctional chemical moiety comprises lysine, said linker comprises (H-Gly-Pro-Lys-Pro-Val-Gly-Nva-Trp-Lys-OH), and said biologically active agent comprises methotrexate.

66. The conjugate of claim 57, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 2,000, said multifunctional chemical moiety comprises lysine, said linker comprises (H-Gly-Pro-Leu-Gly-Pro-Lys-NH_2), and said biologically active agent comprises methotrexate.

67. The conjugate of claim 3 comprising the structure:

$$\mathbf{Q(\text{-}P\text{-}L\text{-}D)_k}$$

in which Q is said common multifunctional chemical moiety, P is said water soluble polymer segment, L is said linker, D is said biologically active agent, and k is an integer greater than or equal to 2.

68. The conjugate of claim 67, in which k is an integer from 2 to about 100.

69. The conjugate of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 4,000, said common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, said linker comprises (H-Gly-Pro-Leu-Gly-Pro-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active agent comprises methotrexate, and said integer is 4.

70. The conjugate of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of about 4,000, said common

multifunctional chemical moiety comprises an 8 arm dendrimer, said linker comprises (H-Gly-Pro-Leu-Gly-Pro-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active agent comprises methotrexate, and said integer is 8.

71. The conjugate of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, said common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, said linker comprises (H-Glu-Glu-Glu-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active agent comprises methotrexate, and said integer is 4.

72. The conjugate of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, said common multifunctional chemical moiety comprises an 8 arm dendrimer, said linker comprises (H-Glu-Glu-Glu-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active agent comprises methotrexate, and said integer is 8.

73. The conjugate of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, said common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, said linker comprises (H-Gly-Pro-Lys-Pro-Val-Gly-Nva-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active substance comprises methotrexate, and said integer is 4.

74. The conjugate of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, said common multifunctional chemical moiety comprises an 8 arm dendrimer, said linker comprises (H-Gly-Pro-Lys-Pro-Val-Gly-Nva-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active agent comprises methotrexate, and said integer is 8.

75. The conjugate of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, said common multifunctional chemical moiety comprises pentaerythritol or a pentaerythritol analog, said linker comprises (H-Gly-Pro-Tyr-Ala-Tyr-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active agent comprises methotrexate, and said integer is 4.

76. The construct of claim 67, in which said water-soluble polymer segment comprises poly(ethylene glycol) with a molecular weight of 4,000, said common multifunctional chemical moiety comprises 8 arm dendrimer, said linker comprises (H-Gly-Pro-Tyr-Ala-Tyr-Trp-Lys(ε-CO-CH_2-CH_2-OH)-NH_2), said biologically active agent comprises methotrexate, and said integer is 8.

77. A pharmaceutical composition comprising the conjugate of claim 1 and a physiologically acceptable carrier.

78. The pharmaceutical composition of claim 77, in which said composition is suitable for injection, or oral, topical, inhalation, or implantation methods of administration.

79. A method of alleviating a pathological condition comprising administering an effective

amount of the conjugate of claim 1.

80. The method of claim 79, in which said pathological condition comprises neoplastic diseases, chronic inflammatory diseases acute inflammatory diseases, cardiac diseases, renal diseases, liver diseases, lung diseases, neurological diseases, musculoskeletal diseases, and immunological disorders.

81. The method of claim 79, comprising regulating cardiac function, renal function, liver function, lung function, or neurological function.

82. The method of claim 79, comprising modulating immunological function.

83. The method of claim 79, comprising modulating hormonal function.

84. The method of claim 79, comprising treating microbial infections.

85. The method of claim 79, comprising regulating scar tissue.

86. A method of making the conjugate of claim 57, comprising:
(i) attaching said biologically active agent to said linker,
(ii) attaching said linker to said multifunctional chemical moiety, and
(iii) attaching said multifunctional chemical moiety to at least two of said water soluble polymer segments.

87. A method of making the conjugate of claim 57, comprising:
(i) attaching said biologically active agent to said linker,
(ii) attaching said multifunctional chemical moiety to at least two water soluble polymer segments, and
(iii) attaching said multifunctional chemical moiety to said linker.

88. A method of making the conjugate of claim 72, comprising:
(i) attaching said biologically active agent to said linker and attaching said linker to said water soluble polymer segment to form a construct, and
(ii) attaching at least two of said constructs to said common multifunctional chemical moiety via said water soluble polymer segment.

89. A method of making the conjugate of claim 72, comprising:
(i) attaching said biologically active agent to said linker,
(ii) attaching at least two of said water soluble polymer segments to said common multifunctional chemical moiety, and
(iii) attaching said linker to said water soluble polymer segments.

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(54) Title: ENZYMATICALLY ACTIVATED POLYMERIC DRUG CONJUGATES

(57) Abstract: The present invention relates to a polymeric drug conjugate with one or more biologically active agents conjugated via an enzymatically cleavable linker to either a regular repeating linear unit comprising a water soluble polymer segment and a multifunctional chemical moiety, or a branched polymer comprising two or more water soluble polymer segments each bound to a common multifunctional chemical moiety, as well as to methods of making such conjugates. The present invention is also directed to pharmaceutical compositions comprising such conjugates and to the use of such conjugates to treat pathological conditions.

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 00/11670

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, EMBASE, BIOSIS, EPO-Internal, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

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'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2000	08/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Berte, M

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/11670

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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A	EP 0 838 224 A (KURARAY CO) 29 April 1998 (1998-04-29) claims ---	1-89
A	EP 0 712 635 A (KURARAY CO) 22 May 1996 (1996-05-22) claims 1-3 -----	

1

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-89 relate to an extremely large number of possible compounds and methods. In fact, the claims contain so many options that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely those compounds and methods recited in the examples and closely related homologous compounds.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interm. ...al Application No
PCT/US 00/11670

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